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CONCERNING THE MECHANISM OF BARTONELLA ANEMIA IN SPLENECTOMIZED RATS

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The characteristics of *Bartonella muris* infection in rats have been reiterated by many investigators, since Mayer's first observation in 1921. (See review by Weinman, 1944.*[1]) It has been shown that as long as the spleen is intact, the disease remains dormant and becomes manifest only after splenectomy. Nevertheless, the complete mechanism of this infection is still obscure.[2] Repeated attempts to relate resistance or immunity to the spleen have failed leaving many questions unanswered. In fact, it would seem today that the experiments in support of the spleen theory were misleading rather than helpful in clarifying the problem.

In our present endeavor to find a more adequate explanation for this phenomenon, it became evident that the "failures" regarding the protective mechanism of the spleen helped us to reach a better understanding.

The results will be presented in five sections.

METHODS AND MATERIALS

The Sprague-Dawley (Madison, Wisconsin) strain of albino rats, which is normally free of bartonella infection, was used throughout these experiments. The animals were received in small groups, as needed, in order to avoid adventitious infestation. Sample rats, taken at random from each group, were checked for latent infection by splenectomy and blood examination. The animals weighed between 240 and 280 g, except for those in the parabiotic series, which were united at a much earlier age (80 to 100 g) for use when they became older. They were kept in separate cages and fed with Rockland rat diet and water offered ad lib.

The bartonella infection was induced in the test animals by intraperitoneal injection, using the highly infected blood of a splenectomized wild rat. Subsequently this particular strain of *Bart. muris* was maintained in a separate group of stock animals.

For testing, conventional hematological and histological technics have been used. The blood smears were treated with Wright's stain; the reticulocytes were counted with brilliant-cresyl-blue (Dameshek's method); the histological preparations were stained with hematoxylin-eosin and Gomori's iron stain. For easier recognition of the very small endocellular parasites, a 10× ocular and a high power apochromatic immersion objective proved most satisfactory (F.d. 1.5 mm, N.A. 1.30; 120×).

The efficiency of the parabiotic union was tested by a method developed recently by Scheff and Plagge.[3]

Animals receiving cortisone were injected with Cortone acetate (Merck) in doses of 5 mg per day subcutaneously.

RESULTS

A. The course of bartonella infection in single animals

Two criteria have generally been used to characterize the course of infection with some degree of consistency: (1) mortality and (2) the development

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* References quoted by Weinman have been omitted from this paper; only those are presented here which have appeared after 1944.

1. Weinman, D. 1944, Infectious anemias due to bartonella and related cell parasites. Tr. Am. Phil. Soc., N.S. **33**: part 3: 290–338.
2. Wigand, R. and Peters, D. 1952, Neuere Untersuchungen über Bartonella muris Mayer. Ztsch. f. Trop. u. Parasitol. **3**: 437–452.
3. Scheff, G. J. and Plagge, J. C. 1955, Use of fluorescein in testing the union of parabiotic rats. Proc. Soc. Exper. Biol. & Med. **88**: 559–561.

TABLE 1.—*Experiments with single carrier rats infected with* Bart. muris.

Number of animals	Latency of infection before splenectomy (days)	Interval between splenectomy and appearance of anemia (days)	Blood findings before and after splenectomy (average and range)		Recovered	Died
			Before	After		
Total: 30	0–335*	2–16	Hb† g%		23	6
(a) Anemia developed in 29 (96.6%)		5(66%)	15.22 (12.67–17.60)	5.55 (2.78–7.20)	(76.6%)	(20%)
			RBC (million/cu mm)			
(b) Anemia did not develop in 1 (3.3%)			9.76 (8.34–11.22)	3.16 (1.60–7.87)		

* Length of this period had little, if any, influence on subsequent anemia.
† Hb readings with Coleman Junior Spectrophotometer.

of anemia. Since mortality percentages based on a total number of splenectomized animals are of little value without the exact knowledge of the carrier rate, the present observations were made on deliberately infected rats. In a series of 30 animals only 6 died (20%) as an immediate outcome of the infection. This is considerably lower than reported by other investigators (variations ranging from 30 to 80%).

At this point of presentation no other explanation can be offered than perhaps the difference in breed and maintenance of the animals. But this question will be taken up in the discussion again with supporting evidence pointing to additional factors.

On the other hand, the second criterion, namely, the anemia with the typical and abundant appearance of the microorganisms in the blood, developed in all (29) but one animal. Hence, the follow up of the course of infection by hematological studies is more reliable than the recording of the death rate. This blood dyscrasia has been described as a progressive hemolytic, macrocytic anemia frequently accompanied by hematuria.[4] Subsequently, most of our animals fully recovered from the anemia and only a few had relapses. Contrastingly, others found this to be the case frequently. In addition, we occasionally came across individual rats which were refractory to bartonella anemia in spite of splenectomy. Perusal of the literature revealed that similar observations were reported before (Schilling et al. 1928, and others). Dinger (1929) furnished a possible explanation when he found an accessory spleen in such a case. However, careful search failed to indicate the presence of an accessory spleen in our study and also in most other investigations.

Table 1 summarizes the pertinent results.

B. Parabiosis experiments

After having verified the trigger effect of splenectomy in provoking the anemia in carrier rats, it became pertinent to investigate the way in which the spleen exerts its restraint on anemia while present in the animal. Scattered reports appear to indicate that the hormonal hypothesis is still prevalent. Parabiosis experiments have frequently been found expedient to test this possibility. However, when applied to this particular problem, the findings in the past as well as in the present have been conflicting.

In this series of experiments a total of 18 pairs of parabiotic rats have been studied. Of these 12 could be considered successful in the sense that the removal

4. Kodicek, E. and Carpenter, K. J. 1950, Experimental anemias in the rat. Blood, **5**: 522–552.

TABLE 2.—*Experiments with infected parabiotic animals.*

Number of pairs* (total)	No anemia after first but severe anemia after second splenectomy (pairs)	Anemia after first splenectomy (pairs)	No anemia after either splenectomy (pairs)	Survived both splenectomies	Died after second splenectomy
18	12	5	1	13(72%)	5(28%)

* Each pair was accompanied by a single carrier animal from the same stock for control, infected and splenectomized jointly with the parabiotic pair. All became anemic after splenectomy.

of the spleen in one of the parabionts had seemingly no adverse effect on either of the twins. At the same time a concurrently splenectomized single animal used as a control invariably developed anemia. Then, after a suitable interval the removal of the second spleen in the alternate parabiont produced an unequivocal severe anemia in both animals. As a result 5 pairs (5/18 = 28%) succumbed during the first wave of anemia, indicating a somewhat higher death rate among parabionts than among single animals, whereas 7 pairs recovered after the anemic phase subsided.

In the remaining 6 pairs of animals unexpected "failures" were encountered. In 4 of them anemia developed soon after the first splenectomy and relapses occurred again after the second spleen was removed. Then, peculiarly, one pair showed severe anemia after the first splenectomy, but no anemia after the second spleen was taken out. And finally one pair remained totally unaffected by either the first or the second operation.

Hence, since the number of "failures" could hardly be considered insignificant, some doubts have arisen with regard to whether or not the spleen alone is responsible for averting the fatal or transient anemia.

Results are summarized in table 2.

C. Reticulocyte counts

In spite of general agreement concerning the increase of the reticulocytes during the active phase of blood regeneration (Perla and Rivero, 1933 and others), a limited number of counts were made in various phases of infection by ourselves to verify this point. It seemed important to study the efficiency of bone marrow function before and after the anemic imbalance following splenectomy.

In the majority of observations the rise began on the 4th or 5th day after operation reaching levels up to 30 to 35% during the active phase and nearly double that height (55%) during the relapses (normal count 2 to 4%).

In fatal cases a precipitous fall of reticulocytes preceded the fatal outcome of the anemia, whereas when recovery took place the drop was more gradual and inverse to the total increase in reticulocyte count.

In a few animals a somewhat different pattern was observed. Either the reticulocyte count was above normal from the start (more than 5%) increasing from that level relatively more rapidly, or on the contrary, the count did not increase as usual following splenectomy. In the first case, either no anemia developed, or remained negligible; in the second case, the anemia began early and became more severe.

Hence, the reticulocyte count reflects a variable efficiency of the bone marrow, which in turn may have a significant bearing on the eventual development and outcome of the anemia.

Table 3 contains the pertinent data.

D. Experiments with cortisone-treated animals

The effect of cortisone has been studied by several investigators on a variety

TABLE 3.—*Reticulocyte counts (percent) in* Bart. muris *infected rats.*

Rat no.	Before splenectomy		After splenectomy (Active phase)							Remarks
	Days									
	7	2	1	3	5	7	9	15	21	
1	3.5	2.5	3.0	5.4	11.2*	32.3	29.0	7:5		
2	4.8	2.0	2.6	8.8	16.0*	35.5	33.3	10.5	55.1	relapse on the 21st day
3	6.2	5.2	5.8	12.7	38.2	45.0	42.0	35.7	28.7	no anemia
4	2.3	1.7	1.0	3.4	6.3	16.0*	22.0	9.8		
5	2.1	3.1	2.0	1.7*	1.4					died on 6th day
6	2.8	4.3	1.6	4.4	9.7*	25.6	33.0	8.1		
7	3.0	3.3	2.1	3.6	7.8*	30.2	35.7	14.5		
8	2.8	2.5	2.3	4.3	10.3*	28.3	26.7	7.9		
9	2.0	2.2	1.8	2.8	3.6*	1.5				died on the 8th day
10	4.0	2.9	4.4	3.7	7.8	23.7*	31.6	12.9		

* Marks the approximate onset of anemia as determined by RBC count and Hb reading.

of experimental infections in recent years.[5,6] In spite of some incongruity in their findings as to minor details, it is commonly accepted by now that cortisone profoundly suppresses inflammation. Therefore, in those conditons in which the inflammation participates in the body's defense against microorganisms, an aggrevation of the infection should result, causing an early bacteriemia and a less favorable survival time.

With this in mind, the effect of cortisone on *Bart. muris* anemia was tested, expecting a more severe course of the infection and a significantly higher lethality. Although these expectations have not entirely materialized, some observations proved most interesting.

The animals were divided into 5 groups, each consisting of 5 rats. Except for one group (group I), which was composed of bartonella-free animals, in all other groups (II, III, IV, and V) infected carrier rats were used. In groups I and II the cortisone was given daily for 24 days prior to splenectomy (5 mg subcutaneously). No bartonellas appeared in the blood smears during this treatment, but a slight but constant increase in RBC and Hb was observed. This being an indication of bone marrow stimulation, it is consistent with former findings. Then, cortison administration was discontinued and 3 animals in each group were splectomized. The observations were pursued further for 3 weeks. Only one of the splenectomized carrier animals suffered a slight anemia and even in this one, the bartonellas did not appear in the blood.

Group III was studied under quite similar cirucmstances but received cortisone (5 mg s.c.) for only 10 days before splenectomy. Blood changes in this relatively short preliminary period did not occur, but contrary to the previous groups (I, II) after splenectomy severe anemia and bartonellosis developed in all animals. One rat died during the anemic spell.

In group IV, the cortisone treatment was started only after splenectomy and then continued for 24 days. As in the

5. Michael, M., Jr. 1952, Cortisone and ACTH in infectious processes. Monographs in Medicine, Ser. 1, 271–294.
6. Montgomery, M. M. Benedek, T. G. and Poske, R. M. 1955, Relation of ACTH and Cortisone to Infection. Med. Clin. N. Am. **39**: 81–94.

previous group (III) severe anemia and bartonellosis developed.

Group V comprised animals which had been splenectomized earlier, developed typical symptoms, then fully recovered. After a variable lapse of time, cortisone treatment was instituted for periods ranging up to 24 days. Shortly, in all animals a severe recurrence of the anemia became manifest with a heavy flare up of the bartonellas in the blood. One of the animals succumbed to the infection, whereas 4 gradually recovered again. Although recurrences of this type sometimes have occurred spontaneously, the inciting effect of cortisone in precipitating the relapse seemed undeniable.

In the course of these experiments the preliminary impression was gained (though more extended work will be necessary to verify it) that cortisone given in the presence of the spleen over a long enough period counteracts the anemia and thereby protects the animals after splenectomy, whereas if the spleen was removed before cortisone treatment, the progress of the infection became rather aggravated.

Table IV comprises the results of this section.

TABLE 4.—*Experiments with cortisone-treated animals. Blood findings (average and range).*

Groups (5 animals in each)	Before splenectomy				After splenectomy				Remarks
	Cortisone				Cortisone				
	Before		After		Before		After		
	RBC‡	Hb gm%	RBC‡	Hb gm%	RBC‡	Hb gm%	RBC‡	Hb gm%	
I (Control) C* daily for 24 days before splenectomy	8.48 8.16–9.24	13.57 12.68–14.68	10.78 9.64–11.46	16.53 15.87–17.10			7.99 7.28–9.06	13.38 10.52–15.38	No anmeia; no B†
II (Infected) C* daily for 24 days before splenectomy	9.91 9.08–10.76	14.89 13.84–15.64	12.00 10.46–13.38	16.69 15.87–17.60			9.18 8.18–10.16	14.28 13.03–15.69	No anemia; no B
III (Infected) C* daily for 10 days before and 14 days after splenectomy	9.01 8.06–10.12	14.54 12.48–15.64	9.88 9.24–10.40	15.51 15.11–16.30			3.70 2.33–5.59	6.07 4.23–9.87	Anemia; B in abundance (1 rat died)
IV (Infected) C* daily for 24 days after splenectomy					10.06 8.16–11.46	14.77 12.48–15.87	3.19 1.57–5.02	3.79 3.00–7.20	Anemia; B in abundance
V (Infected) C* daily for 24 days after complete recovery from first anemic spell					9.71 8.9–10.18	14.94 14.07–16.08	4.16 3.54–5.25	7.42 7.20–7.73	Anemia; B in abundance (1 rat died)

* C, Cortisone. † B, *Bart. muris*. ‡ Millions/cu mm.

Groups I and II served as controls. Group I comprised animals which had been splenectomized earlier, developed typical symptoms, then fully recovered. After a variable lapse of time, cortisone treatment was instituted for periods ranging up to 24 days. Shortly, in all animals a severe recurrence of the anemia became manifest with a heavy flare up of the bartonellas in the blood. One of the animals succumbed to the infection, whereas 4 gradually recovered again. Although recurrences of this type sometimes have occurred spontaneously, the inciting effect of cortisone in precipitating the relapse seemed undeniable.

In the course of these experiments the preliminary impression was gained (though more extended work will be necessary to verify it) that cortisone given in the presence of the spleen over a long enough period counteracts the anemia and thereby protects the animals after splenectomy, whereas if the spleen was removed before cortisone treatment, the progress of the infection became rather aggravated.

Table IV comprises the results of this section.

E. Findings in the spleen

Since the spleen occupies such a central position in this disease entity, it seemed warranted to study this organ more closely.

a. Spleen weight/body weight ratio.— First, an attempt was made to correlate the weight of the spleen with 100 g of the total rat weight. For normal animals this ratio was fairly constant, in average 0.38, showing only little fluctuation (0.356 to 0.446). In carrier rats the average ratio was about the same, 0.39, but with a wide range of fluctuation (0.195 to 0.772). The relative size of the spleen, however, had apparently no bearing on

subsequent clinical behavior. The splenic enlargement, often referred to in the literature, is apparently not a constant feature of this disease. In parabiosis the combined weight of both animals was divided by 2 and correlated as above. In 4 out of 5 pairs of animals, the spleen/body weight ratio was increased in the secondly splenectomized animals (section B), indicating compensatory hypertrophy of the latter spleen. (Ratio: 1st spleen 0.36; 2nd spleen 0.43). These animals developed anemia and bartonellosis only after the second splenectomy. In one pair, which showed a decrease in this ratio anemia developed after both splenectomies indicating some kind of inefficiency. Animals which received cortisone for some time had a low spleen/body weight ratio with lesser fluctuation in noneinfected than in infected rats (0.22; 0.153–0.284).

b. Histological changes.—For the microscopic characteristics of the normal rat spleen, reference is made to the description of other investigators.[7,8] It is therefore proposed to limit these observations only to findings which deviate from the normal.

The spleens of carrier rats showed a reduction of the white pulp (Malpighian follicles) with decreased number of lymphocytes. There seems to be present a more than normal amount of reticular fibers in between the lymphocytes, particularly in the middle zone of the follicles. Occasionally, there are also some reticulo-endothelial cells interspersed, containing variable amounts of debris. In the red pulp the sinusoids and the blood vessels are congested, containing more blood than normally. This is especially well reflected in the preparations stained for iron. At times (not always) deeply staining patches of erythro- and normoblasts could also be seen.

In the spleens of the parabiotic animals roughly the same histological changes described for the carrier animals were found. It should be stressed, however, that no appreciable microscopic differences could be detected between the two spleens. Here also, special staining disclosed a considerably larger than normal amount of iron.

After cortisone treatment—depending on the dosage and length of its administration—atrophy and frequently destruction of the white pulp sets in to a variable degree. In contrast, the red pulp which becomes engorged with blood elements appears to be more conspicuous with nuclear staining. In addition, the increased appearance of patchy hematopoietic centers (consisting of erythroblasts and normoblasts) is most striking. All preparations were heavily loaded with iron.

It would seem, therefore, that the reactions in the spleen are not strictly specific, and are somewhat milder than in acute septic infections. An important point, well known, but frequently overlooked, is the ease with which the rat spleen can be induced to renewed erythropoietic activity.[9] It may very well be that in bartonellosis the hemopoiesis of the spleen is a compensatory response to an increased demand which the bone marrow alone is unable to satisfy.

DISCUSSION

Previous attempts to elicit the mech-

7. Orr, J. W. 1932, The spleen in streptococcal infections: histological study of the changes in experimental infections of the rat. J. Path. & Bact. **35**: 149–160.

8. Baker, B. L., Ingle, D. J. and Li, C. H. 1951, The histology of the lymphoid organs of rats treated with adrenocorticotropin. Am. J. Anat. **88**: 313–339.

9. Thomas, Th. B., Pomerat, C. M. and Frieden, E. H. 1949, Cellular reactions in Hemobartonella infected rats with anemia produced by anti-blood immune serum. J. Infect. Dis. **84**: 169–186.

anism of *Bart. muris* anemia were hampered by the usual assumption that the spleen alone is responsible for the course of the infection. It should be pointed out, however, that earlier claims regarding either hormonal or protective influences exercised by the spleen, have never been fully substantiated in this disease. Furthermore, there are numerous observations which have not been explained by the removal of the spleen per se.

From the evidence now at hand (a) one knows that there are virulence differences among various *Bart. muris* strains, just as there are resistance differences among various breeds of rats and (b) one may assume that in infected animals an interplay of the bone marrow and spleen is maintaining a delicately balanced equilibrium between blood cell formation and destruction. Due to increased demand for red blood cell replacement (infectious anemia), the spleens of rats are particularly ready to revert to their embryonic hematopoietic function (appearance of hematopoietic centers in the red pulp). Hence, if the spleen is suddenly removed, the bone marrow, at normal level of activity, cannot cope with this change immediately, and a more or less severe anemia develops in the majority of cases. If, in addition, the bone marrow is depressed (experimental poisons, X ray)[10,11] the situation becomes more aggravated. On the other hand, if we are dealing with more resistant well fed animals, or if previously the bone marrow has been stimulated (cortisone treatment before spleen removal), then the compensatory function is more efficient and prevents anemia from developing (increased reticulocyte count serves as index). (c) One can explain many previously incomprehensible observations: why there is such a variation in lethality among splenectomized animals; why a variable portion of animals is apt to recuperate as soon as the bone marrow function can adequately compensate for the loss of the spleen; why some carrier animals did not become anemic at all after splenectomy; why a part of the animals relapse (with no relationship to the spleen) if the compensation is not satisfactory; why a fair number of parabiosis experiments failed to meet the expectation not to become anemic while one spleen was still present; and why debilitation (concurrent infection or X ray) without splenectomy is also effective in inducing symptoms[12,13] though much less so than the removal of the spleen.

Open for future speculation is the observation regarding the connection between the spleen and the effect of cortisone. If cortisone was administered before the removal of the spleen the animals escaped anemia after splenectomy. If, however, cortisone was given to previously splenectomized animals, which had recovered from the anemia, an immediate severe relapse ensued.

On the whole, it would seem, the causative agent (*Bart. muris*) is primarily responsible for starting the chain of events but the outcome of the infection will depend (by elimination) on the efficiency of the spleen-bone marrow interplay.

10. Rekers, P. E. 1951, The effect of x-irradiation on rats with and without *Bartonella muris*. J. Infect. Dis. **88**: 224–229.
11. Scott, J. K. and Stannard, J. N. 1954, Relationship between *Bartonella muris* infection and acute radiation effects in the rat. J. Infect Dis. **95**: 302–308.
12. Taliaferro, W. H., Cannon, P. R. and Goodloe, S. 1931, The resistance of rats to infection with *Trypanosoma lewisi* as affected by splenectomy. Am. J. Hyg. **14**: 1–37.
13. Hsu, D. V. M. and Geiman, Q. M. 1952, Synergistic effect of *Hemobartonella muris* on *Pl. berghei* in white rats. Am. J. Trop. Med. **1**: 747–960.

SUMMARY

To elucidate the mechanism of *Bartonella muris* infection various experimental approaches to the problem have been used. The findings seem to indicate that, in addition to the specific properties of the causative agent, the fate of the splenectomized carrier animals is contingent on the ability or lack of ability to maintain a balanced equilibrium in the blood in spite of increased red blood cell destruction. The spleen, while still present, regains its hematopoietic potential and is particularly active in aiding the bone marrow in this endeavor.

POTENTIAL INSECT VECTORS OF VESICULAR STOMATITIS IN WISCONSIN

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Epizootics of vesicular stomatitis occurred in Wisconsin during the years 1937 and 1949. Both of these outbreaks developed during late summer in the northwestern part of the state. The disease spread rapidly, but not uniformly, and it did not occur in equal intensity throughout the area. The degree of infection ranged from the entire herd to only one or two animals. These observations suggest that a blood-sucking insect might be a vector of the disease during epizootics. Recent studies by Ferris, et al[1] indicate that certain dipterous parasites may serve as effective mechanical vectors during epizootics.

Since the occurrence and seasonal incidence of blood-sucking Diptera in the northwestern part of the state was unknown, a survey for these forms was made during the summers of 1951, 1952, and 1953. The purpose of the survey was to determine the occurrence and relative abundance of biting Diptera peculiar to these areas.

PROCEDURE

Collections were made within the areas of the 1937 and 1949 epizootics, specifically in the vicinity of Bruce (Rusk County) and Spooner (Washburn County). Periodic collections of adults were made with light traps and biting adults were obtained from human and animal hosts by means of collecting bottles or sweep nets. A check-list of species was augmented by collecting and rearing immature forms.

FIG. 1.—Battery operated light trap used in survey studies.

The light trap (fig. 1) was operated by 6-volt batteries with sufficient power to maintain a light and fan. The traps were operated from approximately 2 hours before dusk and continued until the battery failed at approximately midnight. In Rusk County during 1951 one trap was operated in permanent location in a village park. During the 1952 survey, one trap was on permanent location in a wooded low-land area and a second trap was located in the village of Bruce in back of the field station. Attempts to relocate the second trap periodically in areas adjacent to plasture land did not prove feasible. In 1953, two traps were operated in permanent locations, one at the edge of a forested area on high land, and the other on the bank of a small river on low land. One light trap was in operation during 1952 and 1953 in Washburn County in a permanent location at the edge of a swamp. Two trap collections were made per week for all locations.

Biting dipterous insects were collected with a

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1. Ferris, D. H., Hanson, R. P., Dicke, R. J., and Roberts, R. H., 1955, Experimental Transmission of Vesicular Stomatitis by Diptera. J. Infect. Dis. 96: 184–192.

sweep net from cattle while at pasture or during milking periods. Engorged insects having entered the dairy barn with cattle were collected from windows and doors. In 1953, in Rusk County, a human host served as a means of attracting and collecting mosquitoes. These collections were made periodically at eight different sites representing open and wooded swamps, permanent standing water, running water, and high relatively dry forested areas not associated with swamps or rivers. Mosquitoes attempting to bite were collected from the host with bottles and nets. Collections were made during a definite time interval as a means of determining relative abundance.

RESULTS

Summaries of mosquito collections are made in tables 1 through 5. In the light trap collections in Rusk County three species of mosquitoes, *Aedes vexans*, *A. sticticus*, and *A. cinereus*, were most abundant. Twelve additional species of *Aedes*, 5 of *Culex*, 3 *Anopheles*, 4 *Culiseta*, 2 *Psorophora* and one each of *Mansonia* and *Uranotaenia* were included in the collections in relatively minor numbers. The seasonal abundance of species was extremely variable during the 3-year period. Collection data for 1951 indicate a primary peak occurrence during midsummer, with a secondary peak at the end of the summer. The seasonal incidence for

TABLE 1.—*Mosquito collections, Rusk County, Wisconsin.*

Species	Light trap collections†					Collections from human host 1953
	1951	1952		1953		
	High land	Low land	High land	Low land	High land	
Aedes						
vexans	41.3*	31.9*	40.8*	24.6	25.6*	1.6
sticticus	20.6*	33.2*	4.9*	60.4	59.3*	82.5
cinereus	7.6*	6.0*	3.0*	2.8	2.2*	5.6
canadensis	0.1*	0.6*	0.0*	0.3	0.8*	0.5
punctor	0.2	0.3*	2.4*	1.3	0.2	0.8
trivittatus	0.0	3.3*	0.0*	4.8	0.9*	2.5
stimulans	0.0	8.3*	0.0*	0.0	0.1*	3.8
fitchii	0.0	0.6*	0.0*	0.0	0.0	1.0
excrucians	0.0	6.8*	0.0*	0.0	0.06	0.9
triseriatus	0.0	0.1	0.0	0.0	0.1	0.0
spencerii	2.5*	0.0*	0.0*	0.0	0.06*	0.0
intrudens	0.6	0.7	1.2	0.04	0.0	0.0
campestris	3.5*	0.0*	1.2*	0.0	0.0*	0.0
dorsalis	0.4	0.0*	1.2*	0.0	0.0*	0.0
implacabilis	0.0	0.02*	0.0*	0.0	0.0	0.0
undetermined	6.3	1.1	6.1			
Culex						
restuans	3.5	0.9*	0.0*	0.7	1.2*	0.0
apicalis	0.6	5.4	6.7	0.6	0.6	0.0
salinarius	1.2	0.06	0.0	0.0	0.4	0.0
pipens	0.0	0.06	0.0	0.1	0.0	0.0
tarsalis	0.8	0.0	0.0	0.0	0.0	0.0
undetermined	2.5					
Anopheles						
punctipennis	0.0	0.1*	0.0*	0.3	0.5	0.0
occidentalis	0.8	0.8	0.0	0.1	0.3	0.0
walkeri	0.0	0.09	0.0	0.0	0.0	0.0
Mansonia						
perturbans	2.4*	1.2*	23.8*	2.9	3.9*	0.9
Culiseta						
morsitans	3.1	2.0	6.1	0.8	3.2	0.0
inornata	0.8*	0.0*	1.2*	0.1	0.2	0.0
impatiens	0.2	0.3	0.0	0.0	0.0	0.0
melanura	0.0	0.02	1.2	0.0	0.0	0.0
undetermined		0.2				
Psorophora						
ciliata	0.0	0.0	0.0	0.0	0.06*	0.0
horrida	0.0	0.0	0.0	0.0	0.0	0.03
ferox	0.0	0.02	0.0	0.0	0.0	0.0
Uranotaenia						
sapphirina	0.2	0.3	0.0	0.2	0.1	0.0
Total numbers	338	4616	164	2719	1500	3379

* Engorged specimens also collected from dairy barns.
† Represented as a percent of the total collection.

TABLE 2.—*Mosquito collections (light trap) Washburn County, Wisconsin.**

Species	Year	
	1952	1953
Aedes		
vexans	16.6	13.2
cinereus	9.9	9.1
canadensis	1.5	0.3
punctor	1.8	1.2
trivittatus	0.05	0.05
fitchii	0.05	0.03
excrucians	0.1	0.1
spencerii	0.1	0.0
intrudens	0.0	0.03
aurifer	0.05	0.03
Culex		
restuans	0.7	0.9
apicalis	3.4	1.4
salinarius	0.05	0.5
pipens	0.05	0.0
tarsalis	0.05	0.07
Anopheles		
punctipennis	0.1	0.2
occidentalis	0.3	0.5
walkeri	9.4	25.6
Mansonia		
perturbans	37.6	31.0
Culiseta		
morsitans	17.7	10.8
inornata	0.42	0.5
impatiens	0.2	0.3
melanura	0.2	0.03
Uranotaenia		
sapphirina	0.0	4.26
Total numbers	1921	7940

* Represented as a percent of the total collection.

the following two years was reversed. In 1952, the peak occurred during late summer, while in 1953 the greatest numbers were collected in early summer. The seasonal incidence of the two principal species followed this general pattern. *A. sticticus* (table 4) occurred in peak numbers only during mid-summer in 1951, late summer in 1952 and early summer in 1953. *A. vexans* followed this general pattern of *A. sticticus* in 1951 and 1952, but in addition to an early summer peak in 1953, there also occurred a late summer peak. Of the relatively minor species, *A. cinereus* was most prevalent during late summer except at the low land collecting site in 1953. *Aedes stimulans* appeared in large numbers (8% of the total catch for the year) during the latter part of July in 1952, but was absent during 1951 and only one specimen was obtained in 1953. *Mansonia perturbans* was collected every year but only during the early summer. *Culiseta morsitans* followed the general pattern, occurring late in the summer of 1952 and early in 1953.

In the Washburn County area, *M. perturbans*, *A. vexans*, *A. cinereus*, and *C. morsitans* were the most abundant species especially during 1952 (tables 2 and 5). In addition, 8 species of *Aedes*, 5 *Culex*, 3 *Anopheles*, 3 *Culiseta*, and one *Uranotaenia* species were collected in relatively small numbers. While *A. sticticus* was one of the principal species in Rusk County, it was not reported from Washburn County. *M. perturbans* was an early summer species, and the seasonal incidence of *A. vexans* fol-

TABLE 3.—*Weekly distribution of mosquito collections.**

Year	Week beginning														Total number
	June				July				August				September		
	8	15	22	29	6	13	20	27	3	10	17	24	1	7	
Bruce															
1951 highland						19.1	35.0	22.6	1.8	3.3	0.8		17.8		338
1952 highland	6.1	7.3	13.4		9.8	4.9	0.0	19.5				39.0			164
lowland		2.2		2.1	2.1	3.6	3.2	3.3		11.6	15.7	49.3		6.8	4616
1953 highland		59.9	14.1	5.7	2.5	5.3	0.8	0.3	1.2	2.3	1.7	6.3			1500
lowland		44.8	17.6	9.1	2.9		1.2	8.1	0.8	1.9	2.5	11.1			2719
Collections from human host		43.4	27.1	7.7	8.8	1.2	3.4	1.2	1.1	1.4	2.5	1.4	0.9		5379
Spooner															
1952	1.6	1.9	16.0	13.3	13.1	4.1	2.7	2.6	10.4	7.6	4.3	8.0	6.0	8.4	1921
1953	3.0	36.3	2.4	24.6	0.8	6.1		0.8	0.9		1.7	7.9	12.8		7940

* Represented as a percent of the total collection.

TABLE 4.—*Weekly distribution of the most prevelent species in Rusk County.**

	Week beginning														Total number
	June				July				August				September		
	8	15	22	29	6	13	20	27	3	10	17	24	1	7	
Aedes vexans															
1951 highland						21.9	39.1	25.7	4.3	2.9	0.4		5.7		140
1952 highland	0.0	0.0	5.0		13.4	8.9	0.0	35.8				38.8			67
lowland		0.0		1.2	1.6	1.1	0.1	2.4		19.3	24.2	45.4		4.7	1474
1953 highland		10.7	32.1	12.5	2.9	12.5	0.0	0.0	1.6	5.5	3.4	18.8			383
lowland		3.0	18.5	4.0	2.5		1.8	14.5	1.2	6.3	7.6	40.9			670
Collections from human host		0.0	0.0	6.9	12.0	3.6	3.6	3.6	1.8	14.5	16.3	34.4	3.1		55
A. sticticus															
1951 highland						27.6	35.3	28.6	0.0	1.9	1.9		4.7		70
1952 highland	25.0	0.0	0.0		0.0	0.0	0.0	0.0				75.0			8
lowland		0.9		0.0	0.3	0.4	0.6	0.0		10.4	14.3	64.6		8.6	1537
1953 highland		91.9	5.9	1.6	0.1	0.2	0.0	0.2	0.0	0.0	0.0	0.0			890
lowland		69.2	19.3	9.4	1.1		0.1	0.4	0.1	0.0	0.0	0.06			1642
Collections from human host		52.5	26.8	7.5	8.4	0.4	2.0	0.6	0.6	0.7	0.2	0.04			2787
A. cinereus															
1951 highland						15.4	7.7	7.7	0.0	7.7	0.0		61.5		26
1952 highland	40.0	0.0	0.0		20.0	0.0	0.0	0.0				40.0			5
lowland		10.8		6.5	1.8	1.4	1.4	6.5		12.6	17.3	33.1		8.6	278
1953 highland		9.1	12.1	0.0	0.0	6.1	0.0	6.1	6.1	3.0	27.2	30.4			33
lowland		36.0	6.7	6.7	9.3		0.0	16.0	5.3	1.3	9.3	9.3			75
Collections from human host		0.0	27.4	9.9	8.0	2.6	8.4	4.7	2.1	2.6	11.6	11.0	11.6		190

* Represented as a percent of the total collection.

lowed a pattern similar to that observed in the Rusk County collections. *A. cinereus* was most prevalent in late summer during 1952, but occurred in early summer and late summer peaks in 1953. In 1953, *C. morsitans* was relatively abundant and was collected early in spring (May), occurred throughout the summer with a high population in July and appeared again in large numbers during late summer (September).

Mosquito collections in Rusk County in 1953 from a human host are summarized in tables 1, 3 and 4. *A. sticticus* was

TABLE 5.—*Weekly distribution of the most prevelent species in Washburn County.**

Species	Week beginning														Total number
	June				July				August				September		
	8	15	22	29	6	13	20	27	3	10	17	24	1	7	
Mansonia perturbans															
1952	0.5	1.2	28.0	29.4	26.5	5.8	5.4	1.5	0.4	0.0	0.0	0.1	0.4	0.5	720
1953	0.0	16.3	6.5	70.0	1.1	4.5		1.3	0.0		0.0	0.3	0.0		2458
Aedes vexans															
1952	0.0	0.0	0.0	0.0	8.2	3.8	1.2	8.5	32.1	14.4	12.5	11.3	4.4	3.8	319
1953	16.1	38.1	1.1	6.1	1.5	3.0		0.2	0.8		6.8	13.7	11.4		1052
A. cinereus															
1952	2.6	7.4	3.7	5.8	4.2	3.2	0.5	1.6	16.9	14.9	10.6	14.3	5.8	8.5	189
1953	0.6	46.4	0.0	3.3	0.0	1.1		0.8	0.6		3.6	15.5	27.4		724
Culiseta morsitans															
1952	0.3	0.0	5.0	7.1	5.0	1.8	1.5	1.3	11.4	17.1	3.5	8.2	15.9	22.4	340
1953	0.5	5.6	1.4	2.8	1.6	28.8		0.7	6.3		2.3	9.3	27.8		860

* Represented as a percent of the total collection.

TABLE 6.—*Check list of Tabanidae, Simulidae and Heleidae collected from Rusk and Washburn Counties.*

	Date collected:
Tabanidae	
Chrysops	
aberrans Philip	June 30–Aug. 18
aestuans van der Wulp	July 1–July 11
callida Osten Sacken	June 30–Aug. 13
carbonaria Walker	June 16–July 27
celeris Osten Sacken	June 18–July 7
cuclux Whitney	June 23–July 5
excitans Walker	June 12–July 11
frigida Osten Sacken	June 12–Aug. 10
inda Osten Sacken	June 13–July 30
mitis Osten Sacken	May 29–July 31
moecha Osten Sacken	June 26–Aug. 13
montana Osten Sacken	June 25–July 26
nigra Macquart	June 4–June 30
sackeni Hine	June 23–Aug. 16
shermani Hine	July 1–Aug. 15
striata Osten Sacken	June 25–Aug. 20
univittata Macquart	Aug. 13
vittata Wiedemann	June 25–Sept. 1
wiedemanni Krober	June 21–Aug. 14
Hybomitra	
affinis (Kirby)	June 12–July 4
epistates (Osten Sacken)	June 10–July 18
hinei (Johnson)	June 26–July 6
illota (Osten Sacken)	June 12–July 24
lasiophthalma (Macquart)	June 12–July 18
metabola (McDonnough)	June 12–June 23
minuscula (Hine)	June 27–July 26
nuda (McDonnough)	June 12–June 26
frontalis (Walker)	July 2 and July 31
trepida (McDonnough)	June 12–July 24
trispila (Wiedemann)	July 1–July 28
typha (Whitney)	June 12–July 20
zonalis (Kirby)	June 10 and June 22
Tabanus	
catenatus Walker	July 16
fairchildi Stone	Aug. 26
lineola lineola Fabricius	June 28–July 7
lineola scutellaris Walker	June 12–July 28
nivosus Osten Sacken	June 17–July 28
stygius Say	June 21
vivax Osten Sacken	June 30–July 18
Stonemyia	
tranquilla (Osten Sacken)	Aug. 3
Simuliidae	
Simulium	
decorum Walker	
jenningsi Malloch	
luggeri Nicholson & Michel	
venustum Say	
vittatum Zetterstedt	
Heleidae	
Culicoides	
biguttatus (Coquilett)	
crepuscularis Malloch	
guttipennis (Coquilett)	
haematopotus Malloch	
obsoletus (Meigen)	
spinosis Root & Hoffman	
stellifer (Coquilett)	
stillobezzoides Folle & Pratt	
variipennis (Coquilett)	
venustus Hoffman	
villosipennis Root & Hoffman	

the most prevalent species collected, with *A. cinereus*, *A. stimulans*, *A. trivittatus* and *A. vexans* occurring in minor numbers. Only 10 species of *Aedes*, *M. perturbans* and *Psorophora horrida* (one specimen) were collected. Biting species (principally *A. sticticus*) were most abundant in actual numbers during early summer particularly the period during the month of June when 71% of the total number was obtained.

In table 1, the species marked with an asterisk were found engorged with blood and collected in barns or from cattle. Fourteen species of *Aedes*, *Culex restuans*, *Anopheles punctipennis*, *M. perturbans*, *Culiseta inornata*, and *Psorophora ciliata* were observed to feed on dairy animals.

A check list of species of Tabanidae, *Culicoides* (Heleidae) and Simuliidae collected in Rusk and Washburn counties is given in table 6. Of the Tabanidae, species in the genus *Chrysops* persisted in appreciable numbers throughout the summer and fall. All other species were limited to a spring and early summer occurrence. Data on the seasonal incidence of Heleidae and Simuliidae were not obtained.

DISCUSSION

Since epizootics of vesicular stomatitis in Wisconsin were reported only during late summer, it would follow that if an insect vector were involved, it would also most likely occur in greatest numbers during that period or shortly before. A total population curve such as in 1952 in Rusk County and species curves similar to those for *A. cinereus* in 1952 or *A. vexans* in 1952 and 1953 and *A. sticticus* in 1952 would conveniently coincide with an epizootic period. A species of relatively little importance in actual numbers during the 1951–53 collecting period (such as *A. stimulans* during 1952 in Rusk County or *C. morsitans* with a heavy late fall emergence in Rusk County in 1952 and in Washburn County in 1953) might, however, also serve as an effective vector.

There was definite evidence from these studies of cyclic occurrences of species. In Rusk County, *A. vexans*

represented 41.3% of the total population in 1951 and only 25% in 1953. *A. sticticus*, however, increased from 20.6% in 1951 to 60% in 1953. *A. cinereus* represented 8% of the population of Rusk County in 1951, and in Washburn County 9.9% of the total population in 1952. In 1953, this species was only 2.6% in Rusk County but 9.1% in Washburn County. The appearance of *A. stimulans* (8% of the total population in 1952) in Rusk County in relatively large numbers indicates that species abundance as well as peak occurrence is extremely variable, and may coincide with and eventually explain the periodic occurrence of the disease.

An attempt to correlate temperature and precipitation within the epizootic and survey years as well as between the two sets of years does not meet with much success. A glance at the following weather data from June 1st to September 6th will suffice to show the reason.

	Precipitation	*Mean temperature*
1937	6.31	68.9 F
1949	16.43	66.9
1951	19.75	64.3
1952	15.2	60.3
1953	13.97	66.9

There is as much difference between the epizootic and survey years as there is within the two groups of years. It seems evident that the factors concerned in the dissemination of the disease by insect vectors are far more subtle than a weather picture.

The absence of *A. sticticus* from collections in Washburn County is significant in the consideration of an insect vector. This was a predominant species in Rusk County, and has been collected from other areas to the east and north of the Washburn collection station. The occurrence of a species peculiar only to the epizootic area might give a clue to the vector involved. However, these studies also indicate that more extensive collections must be made throughout the area before conclusive epizootiological data is obtained.

It is evident from these studies that mosquito collections must be made not only by means of light traps but from human and animal hosts as well. During the 1953 collections, *A. vexans* and *A. sticticus* were the two principal species collected in light traps. *A. sticticus*, however, was the predominant species (83.6% of the total collection) collected from a human host. It will be noted in the check list (table 1) that 5 other species were observed feeding on cattle that were not attracted to man.

The Tabanidae (with the exception of the *Chrysops*) were collected only during late spring and early summer. Although these insects may be potential vectors in the mechanical transmission of the disease, they would appear to be of little importance during a late summer epizootic. The Heleidae and Simuliidae occur throughout the summer. Little information was obtained or is available on the seasonal distribution of these species.

SUMMARY

Collections of blood-sucking Diptera were made during the summers of 1951, 1952, and 1953 in two Wisconsin counties within an area where epizootics of vesicular stomatitis have occurred. These studies indicated that there was considerable variation in seasonal distribution of mosquito species during the years under observation, and in species occurring in two counties within the epizootic area. The seasonal occurrence of Tabanidae do not coincide with a late summer epizootic of the disease, while little information was obtained on the seasonal distribution of the Heleidae and Simuliidae.

EFFECTS OF HYPOXIA ON RATS INFECTED BY *TRYPANOSOMA LEWISI*

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In previous reports from this laboratory[1,2] we have described the adverse effects of hypoxia on the canary infected by *Plasmodium cathemerium*. These adverse effects are characterized by a relapse of latent infections, by markedly increased grades of parasitemia, and by the development of a significantly decreased effectiveness of chemotherapy. The present report involves another infection, viz. *Trypanosoma lewisi* in the domesticated white rat. This study was designed to establish whether or not the principles involved are more or less general rather than being entirely limited and species specific.

MATERIALS AND METHODS

The experimental animals were young albino rats of either sex weighing from 90 to 110 g. The strain of *T. lewisi* was kindly supplied to us by Professor M. G. Lysenko of the Department of Medical Microbiology and was maintained in rats and transferred at weekly intervals by intraperitoneal inoculation.

The decompression chamber with its modifications has been described as to essential details in a previous publication.[3] Food and water were supplied daily to the test animals within the chamber. Trypanosome counts are reported as thousands of trypanosomes per cubic millimeter and were made every other day with blood from the tip of the tail vein of these animals. Counts of erythrocytes and trypanosomes were made by standard methods. Blood films of the experimental rats were dried in air, fixed in methyl alcohol, and stained with Kingsley's stain. The percentages of division or immature forms were determined by counting at least 100 parasites and noting the number of such forms. Trypanosomes with visible evidence of division and the very short forms are both indicative of division and will be considered in this report as being in the category of "division forms." Additional materials and methods will be presented with the discussion of the experimental procedures.

EXPERIMENTAL

Six rats were placed in the decompression chamber and the pressure slowly reduced, during a period of 18 to 24 hours, to a simulated altitude of 19,000 to 20,000 feet. After maintaining these test animals in this hypoxic environment for 4 days they were inoculated with *T. lewisi*. One ml of a saline suspension of parasitized blood containing one trypanosome per "high dry" field was injected intraperitoneally. The same amount of a blood-saline suspension of trypanosomes was administered to 6 control animals which were kept under normal laboratory atmospheric conditions (altitude 860 ft) for the duration of the experiment. In figure 1 are presented the data obtained from these two different sets of atmospheric environments. The values in this figure represent the mean values for the two groups of rats, the mean difference having a P value of less than 0.05 at the peak level of the infection. The parasitemia of the hypoxic rats gradually

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1. Hanson, R. O. and Tatum, A. L. 1952, Drug, host and parasite inter-relationships in the treatment of avian malaria (*Plasmodium cathemerium* in canaries). J. Infect. Dis. **90**: 105–109.
2. Hughes, F. W. and Tatum, A. L. 1955, The effects of hypoxia on infections with *Plasmodium cathemerium*. J. Infect. Dis. **97**: 231–237.

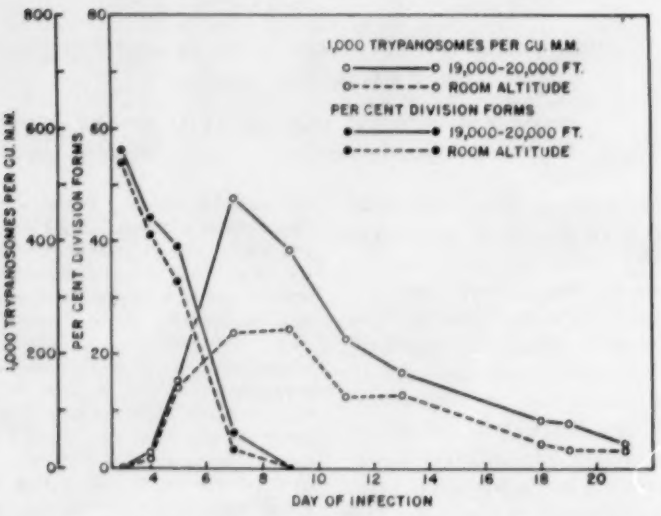

FIG. 1.—Comparative grades of parasitemia of *T. lewisi* in rats at normal room atmospheric pressure and at reduced pressure corresponding to an altitude of 20,000 ft.

returned to much the same level as that of the control rats in the open air of the room. The mean values of the two groups had become essentially the same by the 21st day of infection.

In another series, 6 rats were placed in the low pressure chamber 4 days after the intraperitoneal injections of 1 ml of a suspension of *T. lewisi* (1 ml containing 1 trypanosome per "high dry" field). At the time the animals were placed in the chamber the parasites were just commencing to appear in the peripheral blood in countable numbers. The low pressure chamber was gradually adjusted, during a period of 18 to 24 hours, to effect a simulated altitude of 19,000 to 20,000 ft. Six other rats received the same amount of inoculum and were maintained as controls under ordinary room conditions. A level of parasitemia was reached in the series of "hypoxic" rats which was similar to that shown in fig. 1, where the hypoxia was established prior to inoculation with the protozoal suspension. The peak levels of infection were significantly higher than those in the controls in normal air. In both of these groups the erythrocytic levels of the hypoxic rats increased, within a period of 24 hours after exposure to hypoxia, to a mean level of 7.5 to 9 million erythrocytes per μl of blood, which was maintained at this increased mean level for the duration of the experiments. There appeared to be no direct correlation between the numbers of erythrocytes and of the trypanosomes in the blood, which eliminated the likelihood of hemoconcentration alone being responsible for the augmentation of the parasitemia.

Since maintenance of rats at a simulated altitude of 19,000 to 20,000 ft significantly increased the severity of infection with *T. lewisi*, it was thought desirable to study the effects on this infection of a simulated altitude of still greater magnitude, yet one that would be compatible with life. Each animal of this next group of rats was inoculated with one ml of a suspension of *T. lewisi* containing an average of 1 organism per "high dry" field. As in the previously

described experiments, a group of 6 rats was maintained outside the chamber to serve as controls. On the 4th day of infection the number of trypanosomes in the blood stream was readily counted, at which time 6 rats were put into the chamber and the pressure slowly reduced to a simulated altitude of 22,000 to 23,000 ft. Several noninfected control rats were placed in the chamber along with the test rats in order to check the survival of noninfected rats at this "altitude."

Figure 2 illustrates the results obtained in the experiment just described. The mean degree of parasitemia of the rats in the chamber very rapidly reached a percentage much higher than that of the control rats in the room and the peak of parasitemia was reached on the 8th day of infection in each group. On this day the mean of the numbers of

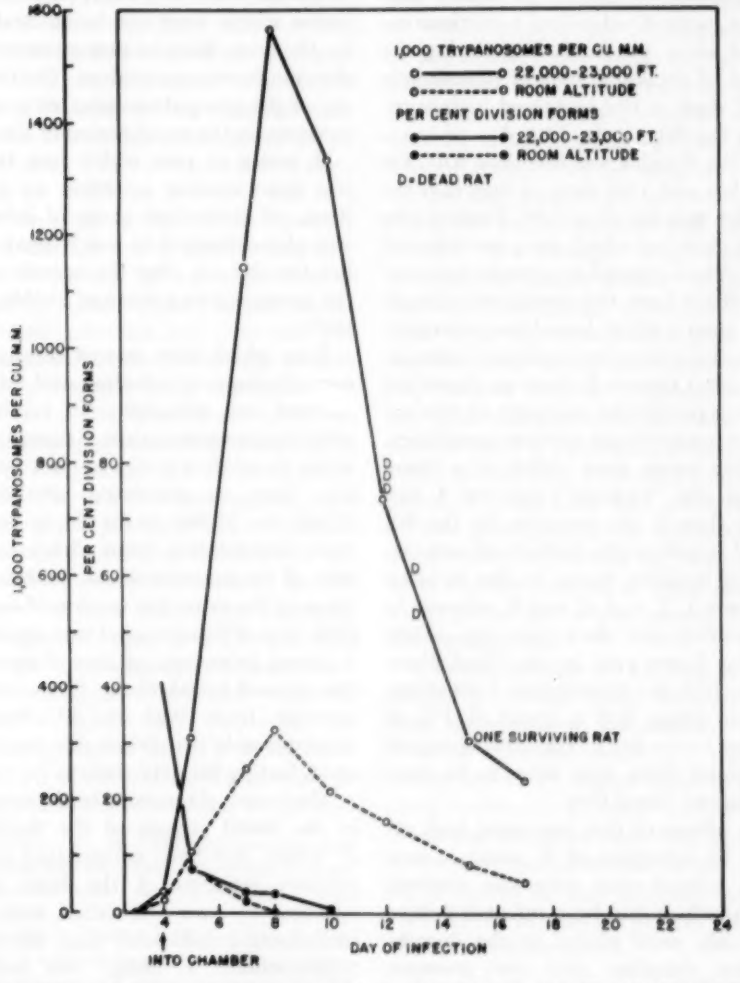

FIG. 2.—Effects of an atmospheric pressure equivalent to that of an altitude of 22,000 to 23,000 ft.

parasites in the blood of these hypoxic rats was above 1550 thousand per μl of blood, while the corresponding value in the controls was 320 thousand per μl. By the 10th day of infection the difference of the levels was still as great but a recovery trend was noted in both groups. Three of the hypoxic rats died on the 12th day of infection and 2 died on the 13th day. These deaths occurred during the phase of mean decline in the grade of infection. It should be stated that rather marked individual variations occurred since 3 animals died during the period of declining level of parasitemia and 2 died at the sustained high level. From the 5th to the 12th day of infection, the *P* value was less than 0.01. On the 15th and 17th days of infection the *P* value was less than 0.05. Control rats in the chamber which were not infected by *T. lewisi* showed no grossly unfavorable effects from this simulated altitude other than a slight initial loss in weight.

The data presented in figure 2 also indicate that there is little or no alteration in the reproductive capacity of this organism under these adverse conditions. Division forms were visible in a blood film of the "hypoxic" rats for 1 day longer than in the controls. On the 8th day of infection the individual percentages of division forms in the hypoxic rats were 1, 2, 4, 4, 3, and 0, whereas in the control rats there were no visible dividing forms seen in the blood films. On the 10th day of infection 1 rat of the hypoxic group had a count of 1% of division forms but in the other 5 rats of this group there was none to be seen in a stained blood film.

The effects of this simulated high altitude on infections of *T. lewisi* in rats at the critical peak were also studied. Six rats which had been infected 9 days previously were placed in the decompression chamber and the pressure slowly reduced, during a period of approximately 24 hours, to a simulated altitude of 19,000 to 20,000 ft. In this group the parasitemia ranged from 60 to 240 thousands per μl of blood at the time of being placed in the decompression chamber. The rat with the highest degree of parasitemia (240 thousands/μl) displayed a gradual increase to 400 thousands/μl by the 10th day and died on the 11th day of infection. The increase in parasitemia was evidently due to persistence of adult (nondividing) forms which were not being destroyed by the host, since no new appearance of division forms was evident. The remainder of the group demonstrated a normal recovery in the usual period of time.

A series of rats which had become free from evident infection for 1 to 6 days, or in the last stage of infection, was also subjected to this hypoxic state but this did not alter the negativity nor the passage into a state of visible negativity.

Rats which have passed through the normal course of infection and have recovered are refractory to reinfection with this same organism. Attempts were made to reinfect 6 rats by first subjecting them to simulated altitudes of 19,000 to 20,000 ft for 24 hours and then reinoculating them with a suspension of trypanosomes and maintaining them at the same low tension of oxygen. Only one of this group of rats developed a patent infection, as was observed in the stained blood films. There was an increase from 0.05 to 50 thousand trypanosomes per μl but this was transient, lasting for only 3 days.

The humoral response to the presence in the blood stream of the flagellate, *T. lewisi*, has been emphasized as the primary reaction of the host, made manifest by two antibodies, one antireproductive (ablastin) and the other trypanocidal.[3] *T. lewisi* also holds a unique position in the group of common

laboratory trypanosomes in its chemotherapeutic responses to arsenical agents.[4] Whether or not the characteristic chemotherapeutic responses of a rat infected with *T. lewisi* are correlative to the distinctive immunological features of this organism is unanswerable at this time.

If rats infected with *T. lewisi* are placed in an environment sufficiently deficient in oxygen, an increased parasitemia occurs if the rats are maintained in this environment for a very few days prior to the inoculation of the parasites, or if they are subjected to a similar grade of hypoxia at the time the parasites are commencing to appear in the blood stream. This increase is of a slight degree at 19,000 to 20,000 ft but of a lethal severity at 22,000 to 23,000 ft. Such a definite rise in parasitemia indicates that either an increased rate of division or a lessened rate of destruction of formed parasites has occurred. The unaltered fall in the percentage of division forms would favor the latter hypothesis, i.e., of interference with destruction of parasites. This temporarily relatively lessened rate of destruction could be due to a decreased formation of a trypanocidal antibody or to a lessened phagocytic capacity of the host. In the previously published studies of the defenses of the host against this flagellate, the phagocytic capacity of the host may have been insufficiently considered.

Reinfection of rats which have recovered or are nearly recovered was apparently impossible by hypoxia alone (20,000 ft) or with hypoxia plus reinoculation, the immune response of the host being too great to be overcome by this additional stress. The titers of ablastin or, the antireproductive antibody, as well as of the trypanocidal antibody, might be of such a magnitude that an inhibition by hypoxia of a suggested third factor, such as the phagocytic capacity of the host, is not great enough to be readily made evident.

The most persistent feature, in all of the experiments involving hypoxia in infections with *T. lewisi*, was the failure to alter the rapid rate of disappearance of the reproductive forms of these protozoa. This occurred in spite of a very significantly increased number of circulating adult flagellates. The formation of ablastin occurs early in the infection and evidently is not altered by this stress factor imposed upon the host. Burnet[5] has strongly implicated the reticulo-endothelial system in the formation of antibodies. If we are to consider that the reticulo-endothelial system has been functionally depressed by the induced state of hypoxia, we can obviously associate the increased parasitemia with a decreased antibody activity as well as a decreased phagocytic capacity of the host. The concentration of the antireproductive antibody evidently was not altered by the hypoxic state but the concept of some selective antitrypanocidal effect cannot be entirely eliminated.

It is not possible at this time to completely generalize these untoward effects of hypoxia on the resultant infections of *T. lewisi* which are reported herein nor of *P. cathemerium* as previously recorded.[2] In both instances, an effect was produced that was injurious to the host and favorable to the invading protozoa so that a higher level of parasitemia resulted from the oxygen-deficient environment.

3. Taliaferro, W. H. 1932, Trypanocidal and reproduction-inhibiting antibodies to *Trypanosoma lewisi* in rats and rabbits. Am. J. Hyg. **16:** 32–84.

4. Kuhs, M. L., Pfeiffer, C. C. and Tatum, A. L. 1936, A study of the curative action of arsenophenylglycine in *T. lewisi* infections in the rat. Am. J. Hyg. **23:** 10–24.

5. Burnet, F. M. and Fenner, F. 1949, The Production of Antibodies, ed. 2, Macmillan.

SUMMARY AND CONCLUSIONS

1. A simulated altitude of 19,000 to 20,000 ft effected an increased level of parasitemia of *Trypanosoma lewisi* in rats.

2. A simulated altitude of 22,000 to 23,000 ft caused the development of a still higher level of parasitemia which was invariably fatal to the tested animals.

3. Reinoculation of rats which had recovered from a normal course of infection did not produce a reinfection even though they were also subjected to hypoxic conditions (19,000 to 20,000 ft simulated altitude).

4. There was no significant alteration in the percentage of division forms of *T. lewisi* in any of the experiments above.

5. Hemoconcentration, due to hypoxia, was not sufficient to account for the increased grade of parasitemia.

EPIZOOTIC *SALMONELLA ENTERITIDIS* INFECTION CAUSING SEPTIC PULMONARY PHLEBOTHROMBOSIS IN HAMSTERS

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Hamsters have been utilized for many years for various purposes in experimental work, but there is little known about their natural diseases. The main references can be counted metaphorically on the fingers of the hand (see Worden, 1947[1]). In the experimental work of our Branch, thousands of laboratory animals are autopsied annually, and since 1952 most of the common diseases have been seen. The present paper is one of a series which have dealt with diseases of laboratory animals and so far have included pulmonary acariasis in monkeys,[2] nutritional muscular dystrophy in rabbits[3] inherited dysplasia of the hip and shoulder joints of rabbits,[4] pulmonary disease in rats,[5] dystrophic calcification of the suprarenal glands in monkeys, cats and dogs,[6] and sarcosporidiosis.[7]

Salmonellosis of laboratory and domesticated animals is well-known to occur in enzootic or epizootic proportions, and in mice and rats it may be a clinically silent, or latent, infection. The salmonellosis studied in hamsters is somewhat unique by virtue of the pathological nature of a fulminating disease with lesions designated in the title. No reference has been found to an identical bacterial infection producing phlebothrombosis in laboratory animals, but there have been observations to an analogous condition in domesticated species (see later).

History of the outbreak and methods

There were two separate outbreaks, occurring several weeks apart, in batches of hamsters which had been bought from commercial establishments. The conditions appertaining to the husbandry of the animal colonies at the source of supply are unknown. In both outbreaks, the animals must have been infected on arrival, because deaths started within a few hours thereafter. Several dead and moribund animals were used for anatomic and bacteriologic study.

All animals (natural and experimental cases) were autopsied in the routine manner; tissues from the lungs and regional pulmonary nodes, liver, kidneys,

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1. Worden, A. N., 1947, U. F. A. W. Handbook on Care and Management of Laboratory Animals. ed. 1, Baltimore, Williams and Wilkins, pp. 196–202.
2. Innes, J. R. M., Colton, M. W., Yevich, P. P. and Smith, C. L. 1954, Lung mites: pulmonary acariasis as an enzootic disease caused by *Pneumonyssus simicola* in imported monkeys. Am. J. Path. **30**: 813–835.
3. Innes, J. R. M. and Yevich, P. P. 1954, So-called nutritional muscular dystrophy as a cause of "paralysis" in rabbits. Am. J. Path. **30**: 555–565.
4. Innes, J. R. M. and O'Steen, W. K. 1956, Splay-leg in rabbits: an inherited disease with similarities to congenital joint dysplasia in children and dogs. To be published.
5. Innes, J. R. M., McAdams, A. J. and Yevich, P. P. 1956, Pulmonary disease in rats. Am. J. Path. **32**: 141–159.
6. Ross, M. A., Gainer, J. H. and Innes, J. R. M. 1955, Dystrophic calcification in the adrenal glands of monkeys, cats and dogs. Arch. Path. **60**: 655–662.
7. Innes, J. R. M. and Eisenstein, R. 1956, Sarcosporidiosis in man and animals. Vet. Annotations and Rev. In press.

spleen and heart and occasionally other organs were prepared by histologic methods. Bacteriological cultures were made from lungs and heart blood, and occasionally from other organs. Subsequent to identification of the microbe, experimental mice, hamsters and rabbits were inoculated with pure cultures of the organism by various routes to determine pathogenicity and to study the pathology.

Nature of the disease

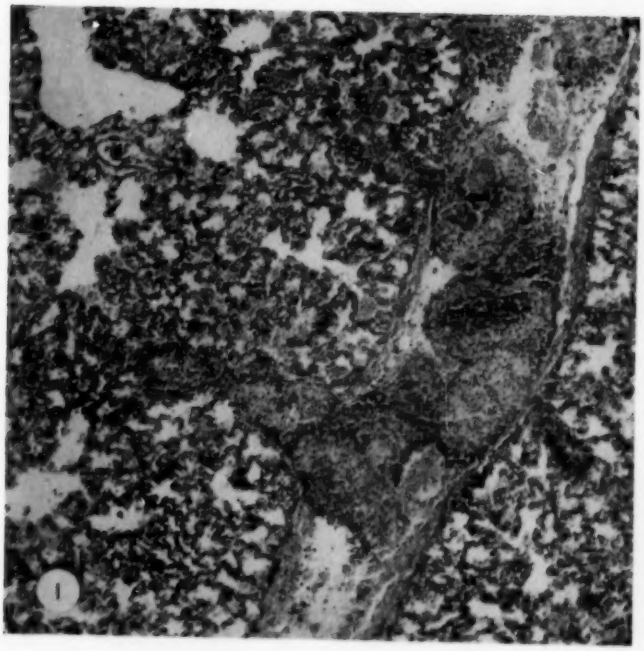
Little is known about the clinical aspects of the natural disease other than that it flared up with startling rapidity, and that within a few days most of the animals in each batch were dead (30 in one, and about 60 in the other). This accords with our subsequent observations on the experimental infection of hamsters and mice.

Pathology

A description in general terms will suffice because the same pattern of tissue changes was seen in all hamsters with the natural or experimental disease, and to some extent in the experimental mice.

In the lung there was no clear macroscopic evidence of any focal, or lobar consolidation, yet morbid anatomy was patently present. The normal pink crepitant appearance of the lungs was lost, and they had a patchy hemorrhagic and partly greyish appearance. The regional lymph nodes were reddened. There were no specific constant gross changes in other viscera of the thorax and abdomen, except for small white foci (necrosis) in the liver. There was no obvious acute enteritis.

Histologically, another story must be told. In the case of the hamsters all lobes of the lungs without exception were affected to the same extent. There were focal areas of congestion, in the middle of which were points of intra-alveolar hemorrhage with pigmentation. The trachea, bronchi and bronchioles were free from inflammation, the lumina containing no secretion, detritus or cellular exudate. Most perivascular lymphatics were dilated and sometimes contained blood or hemolyzed blood. There were focal areas of interstitial pneumonitis and lobular collapse. Pleuritis was seen in one experimental hamster. Above all else, there were lesions involving the veins and venules; the disease process had resulted in septic (partially occluding) thrombi which adhered to the walls and with necrosis and erosion of the latter (fig. 1, 2, 3 and 4). Polymorphonuclear leucocytes and monocytes predominated, and in most thrombi there was fibrin and in some commencing organization. Many of the cells in the thrombi were degenerated, only nuclear fragments remaining. In Giemsa-stained sections organisms were not identified with certainty. The main pulmonary veins, the venae cavae, pulmonary artery, aorta and heart valves, showed no abnormalities and certainly no septic thrombosis. There was an accompanying septic bronchopneumonia in only one animal. The tracheobronchial lymph nodes were congested, hemorrhagic and with sinus catarrh, erythrophagocytosis and occasional necrotic foci (fig. 5); the pericapsular tissue was edematous and showed marked inflammatory cell reaction. Most livers examined showed some degree of focal necrosis (fig. 6 and 7), with inflammatory reaction, which was sometimes very severe, and also venous thromboses. The spleen also contained focal necrotic lesions, but the presence of phleobothrombosis was doubtful. The brunt of the disease process apparently fell largely on the venous side of the pulmonary circulation. There was no apparent primary inflammation of the venular walls, but



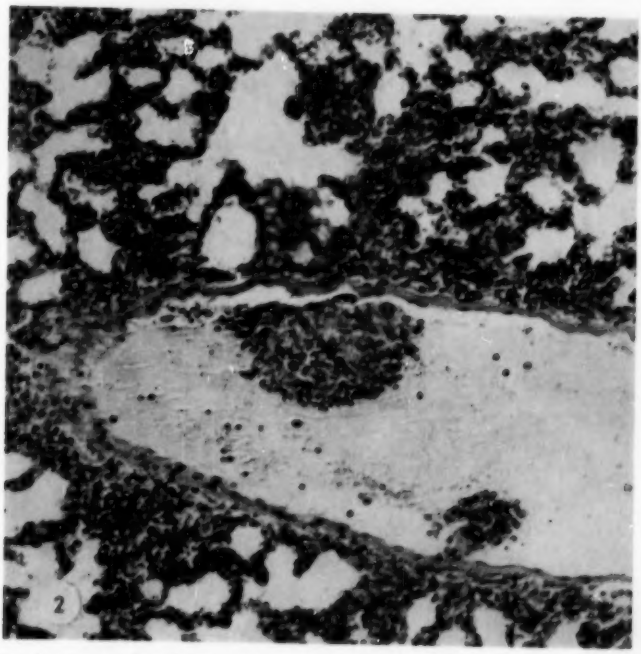
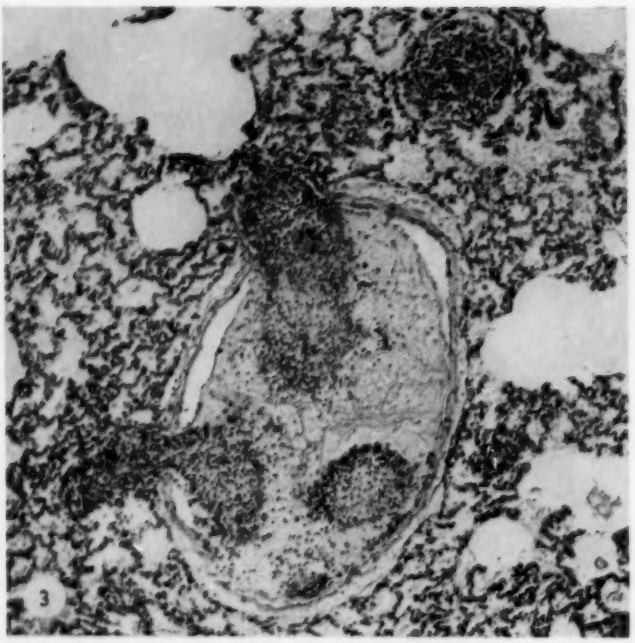


FIG. 1.—Lung, hamster; natural case. Salmonellosis. Showing foci of pneumonitis, hemorrhages and the multiple venous thrombi with erosion of walls. Hematoxylin and eosin. ×125 (591/54).

FIG. 2.—Lung, hamster; experimental infection by mouth. Died in 24 hours. Showing early lesion. Hematoxylin-eosin. ×167 (179/55).



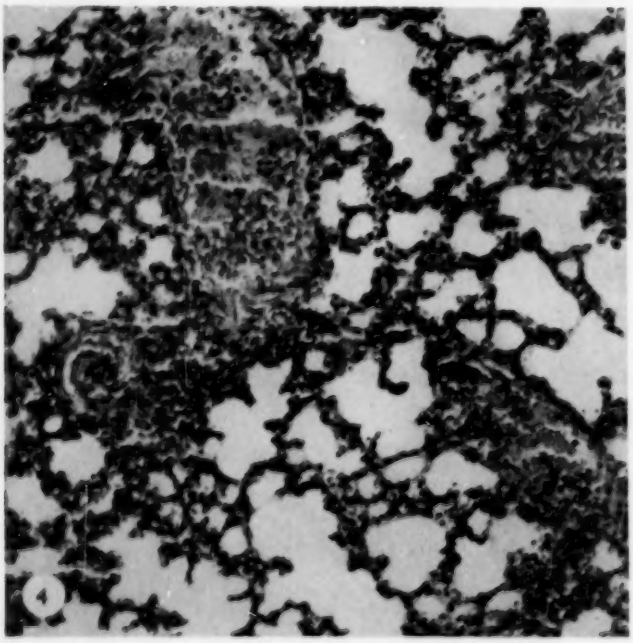
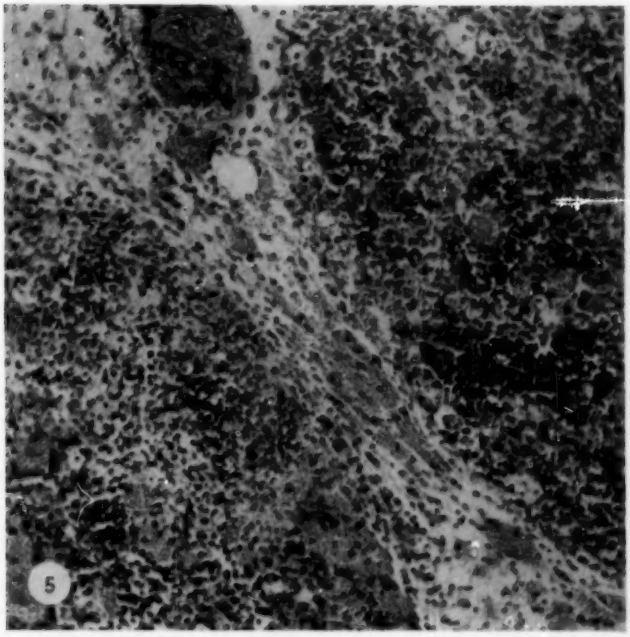


FIG. 3.—Lung, hamster; experimental intraperitoneal infection. Showing same marked features as in figure 1. Hematoxylin and eosin. ×83 (377/55).

FIG. 4.—Lung, mouse; experimental intraperitoneal infection. Died in two days. Showing same features as above. Hematoxylin and eosin. ×167 (201/55).



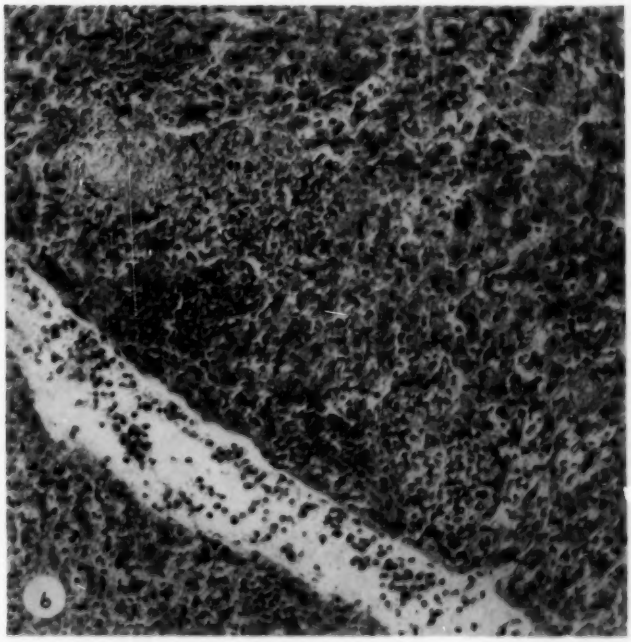
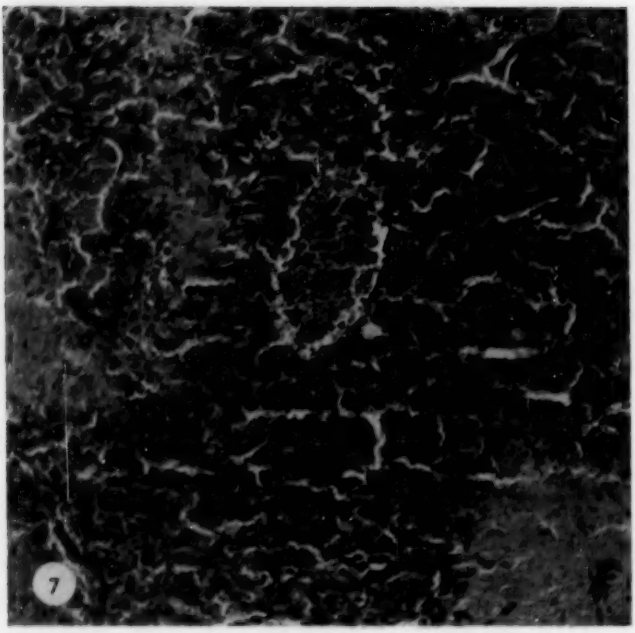


FIG. 5.—Tracheo-bronchial lymph node. Same case as figure 1 showing necrosis, sinus catarrh, erythrophagocytosis, and inflammatory reaction in pericapsular tissue. Hematoxylin and eosin. ×167.

FIG. 6.—Liver, natural case, hamster. Showing necrosis, inflammatory reaction and thrombi in veins. Hematoxylin and eosin. ×167 (4/55).



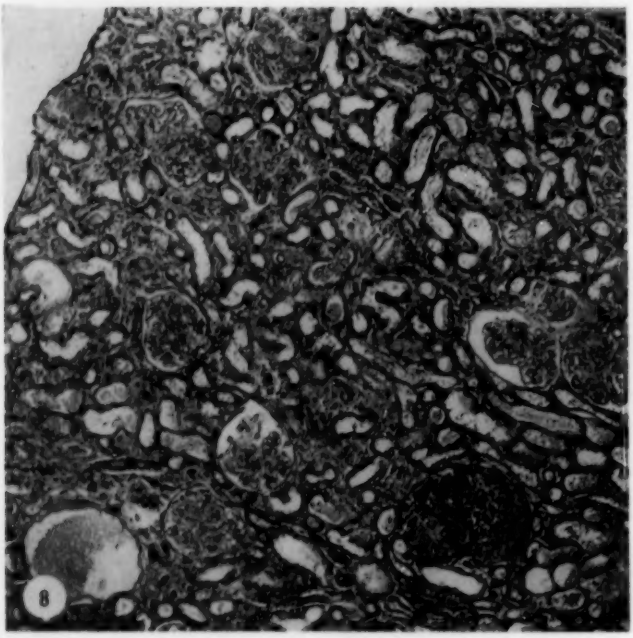


FIG. 7.—Liver, experimental mouse. Same case as figure 4 showing confluent areas of necrosis. Hematoxylin and eosin. ×167.

FIG. 8.—Kidney, hamster; experimental infection. Showing embolic glomerular lesions, superimposed upon a coincidental chronic nephritis (the latter commonly seen in hamsters). Hematoxylin and eosin. ×83 (377–55c).

in those with septic thrombi, cellular reaction extended through to the adventitia. The same phlebothrombosis was found in the experimental hamsters infected by the salmonella recovered (see below), but there were variations in the general pathology from animal to animal, as in any infection. For example, in one hamster which died within 24 hours the venous thrombi contained mainly monocytes, there was an interstitial pneumonitis, and the liver necrosis was less. In yet other hamsters (and mice) infected intraperitoneally, there was peritonitis, perihepatitis and perisplenitis. In some hamsters there were embolic septic lesions starting in the renal glomeruli (fig. 8). In one pregnant hamster, experimentally infected, there were necrotizing lesions in the placenta, and in another animal there was a purulent pericarditis. In hamsters infected by the oral route there was acute enteritis with necrosis of the Peyer's patches.

In the experimentally infected mice variation in severity of lesions was also seen which might be related to the acuity of the disease, and to the time taken for death to occur. In one batch of mice (injected intraperitoneally) all were dead within 24 hours, and there were remarkably few lesions and without septic pulmonary phlebothrombosis. Mice infected by oral routes died with acute enteritis, without any major development of septic venous thrombi, but the latter was seen in the lungs of many mice (fig. 4), although it was never so flagrant as in the hamsters. In mice, focal necrosis of the liver and spleen (fig. 6 and 7) identical to that usually recognized in salmonellosis, was usually prominent.

Bacteriology

The lung and heart blood of four hamsters with the natural disease were cultured, and a pure culture of *Salmonella enteritidis* was isolated from each animal. The strain recovered gave all the typical cultural characteristics, as follows: it did not ferment lactose, sucrose, salicin, or raffinose; it produced acid and gas in glucose, maltose, trehalose and arabinose, and H_2S and nitrites from nitrates, but not indol. There were positive methyl red and citrate reactions, but a negative Voges-Proskauer one. Gelative was not liquefied, and urea was not decomposed. The strain recovered agglutinated with *S. enteritidis* serum; further confirmation of our conclusions regarding the identification of the organism was obtained by submitting a culture to the Salmonella Typing Center, Communicable Disease Center, Chamblee, Georgia.

Experimental work

A pathogenicity experiment was carried out and the following is a précis of the results. Six mice inoculated intraperitoneally with the organisms died within 1 day; of 14 mice infected by the mouth, 3 died during the same day, 1 the 3rd day, 2 the 4th, 3 the 9th day, and there were 5 survivors. Five hamsters injected intraperitoneally were all dead by the 2nd day; of 15 others infected via the mouth, 6 were dead by the 4th day, another 8 by the 7th day and the last hamster died on the 9th day. All the hamsters showed the lesions in varying degrees of severity which have been described above. *S. enteritidis* was recovered in pure culture from the experimental animals, which were examined and cultured. In another experiment 4 hamsters were injected intraperitoneally with the organism, 4 were dosed orally, and 2 rabbits were injected intravenously, mainly for a study of pathology. One hamster in each group was dead within 2 days and one rabbit within 3 days; the other animal were then killed.

DISCUSSION

From the history of the two outbreaks, with almost 100% mortality, it is manifest that the golden hamster is highly susceptible to salmonella, and the experimental work done amply confirms the conclusion. The disease is thus of importance towards establishment and maintenance of healthy breeding colonies. The origin of the infection is unknown, but presumably hamsters could be infected by contact with mice and rats, in which salmonellosis might be latent. Hamster breeding should obviously be isolated away from other rodents, although that statement is gratuitious to those who have experience in maintaining control over infectious diseases. As the disorder is unlikely to be recognized until many deaths have occurred or sections of the lungs examined, its control may lie in prevention, and not attempted chemotherapy.

The phlebothrombotic lesions are of distinct interest. They are most unusual, and have not been recorded before as being caused by any organisms in laboratory animals (see Topley and Wilson, 1946[8] and Hagan and Bruner, 1951[9]). There can be little dispute, however, that the phlebothrombosis is analogous to what has been well documented on salmonellosis of calves in German literature (Nieberle and Cohrs, 1952[10]) as endophlebitis typhosa. A reexamination of sections from a calf which had died from salmonellosis showed no phlebothrombosis in the lungs comparable to that seen in the hamsters, but necrosis and thrombi were present in the liver. We do not know why so-called endophlebitis typhosa occurs in the liver and spleen and not the lungs. The salmonella thrombosis is different from the septic variety described by Stenius[11] in the lungs of horses, in a disease referred to by him as infectious bronchopneumonia, because in the latter both arteries and veins were seemingly involved. It is, however, highly significant, and worthy of emphasis, that an identical picture of thrombosis in lung veins was observed by Morgan (1943)[12] in rabbits following the injection of a toxic somatic antigen of *Eberthella* (now *Salmonella*) *typhosa*.

The venular lesion in the lungs is evidently not an agonal event, for if the latter was the case it would not explain the erosion of the venular wall, and the phlebitis. Consequently, there might be two conclusions. With the onset of the septicemia (which we know occurs and kills in salmonellosis), the bacteria agglutinate on the venular walls to form the nidus for thrombi. Morgan's observations,[12] however, indicate that toxins alone can damage the venous intima and the endophlebitis could form the locus for thrombosis. The lesion is a true septic phlebothrombosis, occurring always in the lungs of hamsters along with necrotizing lesions in the liver and spleen, although the latter are the same as those well defined in murine salmonellosis. The fact that there was a variable incidence of pyaemic lesions in other organs is not surprising.

8. Topley, W. W. C. and Wilson, G. S. 1946, Principles of Bacteriology and Immunity, ed. 3, revised by A. A. Miles and G. S. Wilson. Baltimore, Williams and Wilkins.
9. Hagan, W. A. and Brunner, D. W. 1951, The Infectious Diseases of Domestic Animals, ed. 2, Ithaca, N. Y., Comstock Publishing Co.
10. Nieberle, K. and Cohrs, P. C. 1952, Handbuch d. spez. path. Anat. d. Haustiere., ed. 3, Fischer, Jena, pp. 50, 82 and 368.
11. Stenius, P. L. 1951, The mode of appearance of equine infectious bronchopneumonia (*Bronchopneumonia infectiosa*) and the pathologic anatomical and histological changes in the disease. Skan. Veterinärtidskrift. **5**: 65–94.
12. Morgan, H. R. 1943, Pathologic changes produced in rabbits by a toxic somatic antigen of *Eberthella typhosa*. Am. J. Path. **19**: 135–145.

In conclusion, the hamster could be a useful animal to study the morbid process of phlebothrombosis by experimental infection with salmonella, and also a susceptible species complementary to the mouse for work with salmonella. Since the two main outbreaks occurred, a few other cases have been diagnosed in hamsters; the disease should thus be recognized as an entity important to those in charge of breeding colonies.

SUMMARY

An epizootic disease, with acute course and death, caused by *Salmonella enteritidis* occurred in hamsters. Necrotizing foci were found in the liver and spleen, but the outstanding lesion was a septic partially occluding phlebothrombosis in the lungs, and not necessarily accompanied by bronchopneumonia. Enteric signs and lesions did not dominate the picture. The disease was reproduced experimentally in hamsters (and mice) by intraperitoneal, or oral, infection by the *S. enteritidis* cultured and the organism again recovered by culture.

PARATIDO-STOMATITIS IN CALVES

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With the introduction of rigid quarantine measures for the control and eradication of foot-and-mouth disease in some advanced countries, conditions simulating this infection have gained considerable importance, since an early and correct diagnosis of this disease goes a long way in the success of control measures. Several reports are available from various countries about the incidence of stomatitides and of the methods employed to differentiate them from foot-and-mouth disease (Olitsky, Traum and Schoening, 1926; Traum, 1936; Mason and Neitz, 1940). In India, no such studies appear to have been made so far, firstly, as no records are available regarding the incidence of diseases similar to foot-and-mouth disease of cattle, and secondly, because the comparatively innocuous nature of this disease in Indian cattle does not call for an early differential diagnosis. In this paper, therefore, for the first time in India, it is proposed to record outbreaks of an infectious disease in calves which resembled foot-and-mouth disease, but which, on subsequent transmission experiments, were found to be different.

DESCRIPTION OF THE DISEASE

An outbreak of this disease occurred amongst calves of the college dairy herd in February 1953 and the first clinical case was reported in a Hariana heifer calf, no. 37. This animal, about 6 months old, was recieved at the College Veterinary Hospital with the history of having a swelling at the region of the parotid salivary gland, a slight inappetance and fever, with a temperature of 104 F. Smears from the peripheral blood on microscopic examination showed no bacteria or any other causal agent. On the following day, the temperature had come down to normal and the swelling had become more circumscribed and hard in consistency. On examination of the mouth, two shallow ulcers, each about an inch in diameter, were seen behind the incisor teeth and in front of the carunculae sublinguales. Two more ulcers, both about a centimeter in diameter, one at the root of the right corner incisor and the other in front of and near the central incisors, were also seen. These ulcers were discrete with a distinct red outline and a greyish base. There were no ulcers either on the tongue or on the lips. Salivation was only slight with no indication of frothing at the mouth. There were no lesions on the feet and the animal was feeding normally.

Results of transmission experiments (vide infra) gave the indication that the disease was infectious and a virus was responsible for the production of the lesion. While these experimental studies were being conducted, the farm staff were requested to bring to our notice the occurrence of any further cases in the herd. More cases of the disease could not be discovered until we ourselves decided to examine all the animals in the herd individually. While none of the animals showed any systemic disturbance or the involvement of the parotid salivary gland, mouth lesions indistinguishable from those of the first case

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were discovered in 20 calves ranging in age from 3 months to 2 years (see table 1). No cases were detected in adult stock, including buffaloes, or in calves below 3 months of age. These animals showed ulcers in the mouth behind the incisors, on the upper and the lower lips and on the dental pad, but none were discovered on the tongue or the feet. A second examination made a week after the first revealed 10 fresh seizures and a tendency towards healing up of the ulcers of most of the cases discovered previously. Thus, in one outbreak, out of 101 calves of the susceptible ages at the farm, 32 were found to have acquired the infection, giving a percentage figure of incidence as 31.7. The normal duration of the disease varied from 12 days to a month, as judged from the appearance of the lesions when examined periodically. A notable feature of the outbreak is its specificity to bovine calves, as buffalo calves living in close association with the former, and other ruminants like sheep and goats maintained on the same premises presented no evidence of the disease. The parotid salivary gland involvement, as possessed by the first clinical case, did not appear as a clinical feature of the cases brought to notice subsequently.

TABLE 1.—*Incidence of the disease in calves during the two outbreaks in the farm.*

Age groups of calves	Total number of calves	Number of calves affected
First outbreak		
Calves below 3 months	48	0
Heifer calves above 3 months and below 6 months	8	2
Heifer calves above 6 months and below 2 years	22	10
Male calves above 3 months and below 6 months	20	5
Male calves above 6 months and below 2 years	30	14
Calves suffering from ringworm over 3 months and below 1 year	8	0
Calves maintained for experimental hormonal work	13	1
Total	101 (excluding calves below 3 months)	32
Second outbreak		
Calves below 6 months and above 3 months	28	8
Calves above 6 months and below 1 year	53	14
Buffalo calves below 1 year	27	0
Total	81	22

A second outbreak of this disease occurred at this farm during the months of April and May 1954, and it was brought to our notice when the disease was subsiding. In this outbreak 22 calves, below 1 year and above 3 months of age, were affected giving a morbidity percentage of 22.7 (see table 1). In this outbreak, like that of the previous year, the disease did not affect other ruminants or the adult bovine stock of the farm. The nature of the disease and the disposition of ulcers corresponded with those observed during the first outbreak.

TRANSMISSION EXPERIMENTS

Material and method.—The materials used for the transmission experiments were derived from the mouth ulcers and the parotid salivary gland of the natural and the artificially infected cases. Two methods were employed for the preparation of the ulcer material. They were either the repeated washings of the ulcers with the physiological saline or scrapings from them. Washings from the ulcers and the scrapings from them triturated with the physiological saline were passed through a Seitz filter and the bacteria-and-cell-free filtrate constituted the inoculum. Materials from the parotid salivary gland were the biopsied pieces from calf no. 37, a natural case of parotiditis, and subsequently, apparently healthy pieces from calf no. 32, infected artifically, in which only the mouth ulcers had developed. These were triturated with sand in pestle and mortar, and the cell-free filtrate obtained by passing the triturate suspension in physiological saline through Seitz filter was used as inoculum. The abscess of the parotid salivary gland on culture yielded two species of staphylococcus, *Staphylococcus aureus*, a nonhemolytic strain of *Staph. albus*, and a strain of *Bacterium coli*. A thick mixed suspension of these organisms were also prepared for transmission experiments with a view to reproduce lesions in the parotid salivary gland.

Transmission experiments with healthy bovine calves, using natural infective materials

1. Bacteria-free filtrate prepared from the washings of mouth ulcers of

TABLE 2.—*Results of transmission experiments conducted in calves.*

Description and no. of calf	Sources of the bacteria free inoculum, dose given and method of inoculation	Time taken to produce ulceration	Location of ulcers	Duration of the diseases
Hariana Male calf no. 28	Ulcer washings from the natural case, 5 ml intravenously	3 days	L.* 1 large ulcer	16 days
Hariana Heifer calf no. 41	Ulcer washings from the natural case, 0.5 ml m.m. of the mouth of the commissures	18 days	L, 2 ulcers	12 days
Hariana Heifer calf no. 32	Parotid salivary gland from the natural case, 5 ml intravenously	2 days	L, 2 ulcers; D, 2 ulcers, 2 at the roots of the corner incisors	Destroyed
Buffalo Male calf no. 10	Parotid salivary gland from the natural case, 0.5 ml m.m. of the tongue		No reaction	
Hariana Heifer calf no. 40	Ulcer washings from calf no. 32, 2 cc injected into the left parotid salivary gland	2 days	L, 2 ulcers; U, 2 ulcers; P, 2 ulcers	30 days
Hariana Heifer calf no. 30	Ulcer washings from calf no. 32, 5 cc given intravenously	2 days	L, 1 ulcer; U, 2 ulcers; P, 2 ulcers	28 days
Hariana Heifer calf no. 29	Parotid salivary gland material from calf no. 32, 5 cc given intravenously	2 days	L, 3 ulcers; U, 1 ulcer, 1 at the left corner incisor	24 days

* L, m.m. of lower jaw behind the incisor teeth.
U, m.m. of the upper lip.
D, m.m. of dental pad.
P, m.m. of the lower lip.

the first natural case, calf no. 37, was injected into 2 healthy calves, no. 41 and 28, both about 6 months old. Calf no. 28 was injected with 5 ml of the filtrate intravenously, and no. 41 was injected with 0.5 ml of the same material into the mucous membrane of the mouth, at the commissures. *Results.*—Calf no. 28 showed only one ulcer about ½ inch in diameter behind the incisors on the 3rd day after infection, and this increased to 1 inch in diameter by the end of the week, after which it started healing. The lesion was completely healed by the 17th day after inoculation (see table 2). Calf no. 41 showed a rise of temperature to 104 F on the 11th day after infection, and this continued until the next day. This calf showed two discrete ulcers, each about 1 cm in diameter behind the incisors, 18 days after infection. These increased to 1 inch in diameter after 3 days and healed up 12 days after they had appeared.

2. In this experiment, in order to study whether the virus was carried in the parotid salivary gland of calf no. 37, cell-free filtrate from the triturated pieces of this gland was injected intravenously to calf no. 32 in a 5 ml dose. *Results.*—The calf showed a temperature of 103 F on the next day after infection, and this subsided on the following day. On the 3rd day after infection, two large ulcers, each about 1 inch in diameter, appeared behind the incisors and in front of the carunculae sublinguales. On the 4th day a few more ulcers (fig. 1) were seen near the roots of the corner incisors and the dental

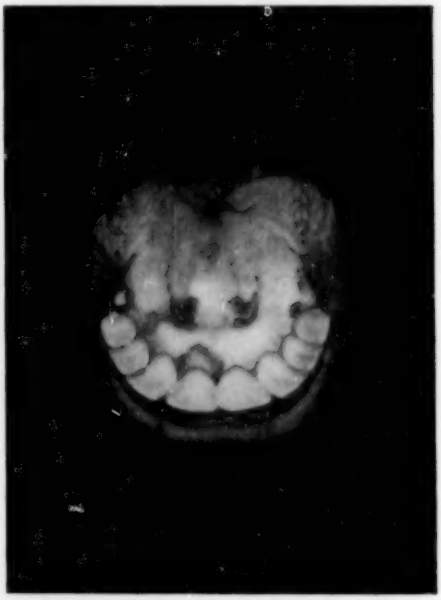

FIG. 1.—Part of the lower jaw, showing ulceration of mucous membrane behind the incisors.

pad. This animal was autopsied on the 6th day after infection, and scrapings from the ulcers and pieces from the parotid gland were collected for further transmission experiments.

Transmission experiments with healthy bovine calves, using artificial infective material

1. The bacteria-free filtrate prepared from the ulcer scrapings of calf no. 32 was injected into two healthy calves, no. 30 and 40. Calf no. 30 was given 5 ml of the filtrate intravenously, while no. 40 was injected with 2 ml of the filtrate into the left parotid salivary gland. *Results.*—Calf no. 40 developed 2 shallow ulcers just behind the incisors on the 3rd day after infection. On the 5th day these ulcers had coalesced to form an extensive ulcerated area, and 4 new ulcers, 2 on each lip, had also developed. These ulcers completely healed up 1 month after infection. Calf no. 30 showed a single ulcer on the lower lip on the 2nd day after infection. On the following day, 2 more ulcers, one on each lip, were seen. These ulcers appeared distinctive on the 5th day, after which they started healing. The lesions healed completely by the 28th day after infection.

2. Five ml of the cell-free filtrate prepared from pieces of the parotid salivary gland of calf no. 32 were injected intravenously into calf no. 29. *Results.*—The calf showed 3 distinct ulcers behind the incisors after the 2nd day of inoculation. One more ulcer developed on the upper lip on the 3rd day, and by the 5th day an extensive ulcer was found behind the left corner incisor. These ulcers healed about 24 days after their appearance.

Experiments to transmit the disease to adult cattle, buffalo calf, yearling sheep and goats

The object of these experiments was to test the infectivity of the virus to adult cattle, buffalo calves and sheep and goats. For this purpose, 2 adult cows about 5 years old, 2 rams and 2 bucks each about 1 year, and 2 buffalo calves each about 6 months old were given pooled virus (obtained from the parotid gland of calf no. 32 and ulcer washings of several natural cases) in doses of 4 ml for cattle, 3 ml for sheep and goats, and 5 ml to buffalo calves, intravenously. The same material was given intravenously to bovine calves no. 40, 29 and 30. *Results.*—While the bovine calves showed the usual ulceration of the mouth, the other animals in the experiment did not show any reaction or ulcer formation, even after a month of observation.

Experiments to transmit the disease to guinea pigs

Five healthy guinea pigs, 3 males and 2 females, were used for this purpose. Three of these were injected intradermally into the foot pad with 5 ml of the pooled virus, while the other two were given 0.25 ml of the same material into the parotid salivary gland. Three bovine calves given the same material intravenously served as controls. While the control calves showed the usual ulceration of the mouth, the guinea pigs did not show any reaction.

The lesions in the parotid salivary gland in the spontaneous case, no. 37, had manifested in the form of an abscess, and the bacteriological examination of the pus had resulted in the isolation of *Staph. aureus*, a nonhemolytic *Staph. albus*, and a strain of *Bact. coli*. As the foregoing transmission experiments with the bacteria-and-cell-free filtrate had failed to reproduce an observable lesion in the parotid gland, in our further attempts to reproduce this lesion, therefore, it was decided on the analogy of several well-known viral infections of animals in which bacteria and

viruses both play causative roles, to investigate the possible etiological significance of these organisms in association with the virus.

Experiments to reproduce the lesion in the parotid salivary gland

Calves no. 54/32 and 55/32 were each injected with 3 cc of the pooled virus (obtained from 3 experimental calves and 6 natural cases of the disease) into the parotid salivary gland. The animals showed no lesions in this gland, except a mild local reaction at the site of inoculation, but the characteristic mouth lesions developed behind the incisors, dental pad and the commissures of the mouth on the 5th day after inoculation. Young broth cultures of *Staph. aureus*, *Staph. albus* and *Bact. coli* isolated from the original case, calf no. 37, were rubbed over the ulcers and sprayed into the mouth, every 4th day, at least thrice during the period the experiment lasted. In another experiment, calf no. 40, an artificially infected case showing mouth ulcers resulting from inoculation of Seitz-filtrate of ulcer washings from case no. 32, was inoculated with 1 ml of the mixture of broth culture of these organisms into the Stenson's duct. *Results.*—The animals were kept under observation for a month, but no inflammatory changes were noticed in the parotid salivary gland of the calves thus treated.

Details of the transmission experiments and the results obtained have been summarized in table 2. The foregoing experiments show that the disease under study is reproducible to healthy calves by inoculating into them the infective materials obtained from diseased calves. The infective material is a virus recoverable from the ulcers in the mouth and the parotid salivary gland of the affected animals, and can pass through EK pads of a Seitz filter. For artificial reproduction of the disease, the routes of inoculation tried are (1) the oral mucous membrane, (2) parotid salivary gland and (3) the jugular vein, and they all have proved successful. The incubation period of the disease reproduced artificially was shorter when the infection was given by the intravenous route, which may probably be due to the larger dose of the virus (5 ml) given by this route. In all these cases the virus had produced ulcers on the mucous membrane of the mouth, including the dental pad and the lips, but in no case did any ulcers appear on the tongue or on the feet. Except in one case, there was no thermal reaction prior to the appearance of the ulcers and the animals showed no systemic disturbances. None of the experimental animals, however, showed the involvement of the parotid salivary gland, as was found in the first natural case.

The virus obtained from the natural case was able to set up a similar disease in healthy calves, and from these artificially infected cases, the virus was recovered both from their ulcers and the parotid salivary gland, which again produced similar condition in healthy calves when inoculated into them. These results may well satisfy the well-known Koch's postulates in establishing the viral etiology of this disease insofar as the lesions in the mouth are concerned. The adult cattle, sheep, goats and the buffalo calves proved refractory to artificial infection with the virus. Guinea pigs did not prove susceptible to intradermal inoculation of the virus in the foot pad.

IMMUNOLOGICAL BEHAVIOR OF THE VIRUS

In view of the fact that this disease was caused by a virus and that its characteristic feature was the development of ulcers in the mouth, at least two aspects of immunological behavior of the virus presented themselves for study, i.e., (1) immunity response in recovered

animals and (2) cross immunity tests with the virus of foot-and-mouth disease.

Such studies are clearly indicated in fulfilment of one of the postulates of Koch, as a positive immunity response to reinfection of the recovered animals would provide a definite proof to the specific role of the virus in causing the disease; and the cross immunity experiments with the virus of foot-and-mouth disease would serve to decide the question as to whether the present aphthous condition of the bovine species in this country could be identified with foot-and-mouth disease or whether it constitutes altogether a separate disease entity.

While the new viral disease was subsiding, with 10 fresh seizures having been reported on April 8, 1953, a natural outbreak of what appeared to be foot-and-mouth disease made its appearance amongst the farm animals on April 22, 1953. Some calves recovered from the new viral infection contracted foot-and-mouth disease, and this finding may perhaps be viewed as a safe criterion in recognizing the new viral infection as a distinct disease entity. Against this, however, is the immunological distinctiveness of the various types of the foot-and-mouth disease virus, and this, taken in conjunction with the close resemblance of the lesions in the two diseases, provided a strong indication for carrying out the cross immunity tests between this virus disease and that of the foot-and-mouth disease. Further, it may have to be admitted that all diseases of cattle, sheep and goats characterized by inflammatory changes on the mucous membrane of the mouth must involve a careful experimental work for establishing a diagnosis with a view to eliminate a serious scourge like foot-and-mouth disease.

Immunity response in recovered animals.—For this purpose, Hariana heifer calf no. 28, which was infected with the virus on February 23, 1953 and had recovered from this disease completely by March 19, 1953, and Hariana calf no. 41, which was infected on February 20, 1953, and had recovered on March 18, 1953, were utilized. Calf no. 41 was injected intravenously with 2 ml of the virus recovered from calf. no. 32, and calf no. 28 was given 3 ml intravenously of the pooled virus (see above). *Results.*—Both these animals did not show any reaction or ulcer formation even when kept under observation for a month.

Cross immunity tests between the virus of foot-and-mouth disease and the new virus.—Of the 4 animals ultilized for the purpose of these tests, 2 had recovered from the new viral infection and 2 from foot-and-mouth disease. The test materials were introduced into the animals by scarification and intravenously to test the infectivity of foot-and-mouth disease virus and the new virus, respectively. The results of the test are summarized in table 3.

Reactions.—A detailed account of the reactions elicited by the calves are noted below. (1) Hariana heifer calf. no. 30.—A rise in temperature to 106 F on the 3rd and 4th day after exposure occurred, and a large vesicle appeared on the tongue on the 3rd day, which ulcerated on the following day. On the 5th day, lesions appeared in the interdigital space of the left fore and right hind limbs, with another ulcer on the tongue. The calf recovered from this attack after a fortnight. (2) Scindhi bull calf. no. 55/33.—A rise of temperature commenced on the 3rd day after exposure, which continued for 3 days and reached a maximum of 106 F on the 4th day. One vesicle appeared on the tongue on the 3rd day, which ulcerated on the following day. Lesions in the interdigital space appeared in the left fore limb on the 4th day. All four limbs were involved by the 6th day, when several ul-

TABLE 3.—*Results of cross immunity tests with foot-and-mouth disease virus and the new virus.*

Animal no. and description	Previous history	Date of exposure to test virus	Method of exposure and nature of virus	Results
Hariana heifer calf no. 30	Recovered from artificial infection of new viral disease	5/31/53	By scarification of the tongue with foot-and-mouth disease virus collected from oral ulcers of 3 natural cases	Positive (suffered from foot-and-mouth disease)
Scindhi Bull calf no. 55/32		4/29/53		
Scindhi Bull calf no. 51/12	Recovered from a natural attack of foot-and-mouth disease	5/ 6/53	5 cc intravenously of pooled new virus collected from the ulcer washings of 3 artificial and 6 natural cases and filtered	Positive (typical oral ulcerations observed)
Scindhi Bull calf no. 48/32		5/ 6/53		

cers on the tongue, dental pad, and the lips had also appeared. The calf recovered from this attack in a fortnight. (3) Scindhi bull calf no. 51/12.—A small ulcer appeared on the level of the right corner incisor by the 10th day after exposure, and the animal recovered after 3 weeks. (4) Scindhi bull calf. no. 48/31.—An ulcer about ½ inch in diameter near the left corner incisor developed on the 4th day after exposure. Another ulcer appeared on the 14th day, and the animal recovered after 3 weeks.

DISCUSSION

The present paper records the occurrence of outbreaks of a new viral disease in calves characterized by ulcerative lesion in the mouth, slight salivation and rise of temperature in some cases. Except in the first natural case, which commenced with the involvement of the parotid salivary gland, in all artificially infected cases, either with the filtrates prepared from the scrapings of the mouth lesions or from the biopsied parotid salivary gland material, this organ remained free from any lesion. In all other natural cases, also, this feature of the disease was not noticed. In view of the fact that in several passage transmission experiments conducted with the parotid salivary gland material, the characteristic mouth lesions were invariably produced, the authors are inclined to the view that the virus of this new stomatitis disease remains localized in the parotid salivary gland. It appears that in nature the infection is acquired through abrasion wounds inflicted on the buccal mucosa when calves of the susceptible age groups are changed from milk diet to roughage fodder. It is highly probable that the virus when discharged in the mouth through saliva may gain entrance into the system through these abrasion wounds.

Parotido-stomatitis as a label for this disease has therefore been coined on the basis of the supposed pathogenic role of the parotid salivary gland in the production of the lesions of stomatitis.

The organisms isolated from the lesion of the parotid salivary gland are of a secondary importance, but what pathogenesis is involved in its causation remains unexplained for the present.

Transmission experiments have shown that the disease is reproducible by introducing the virus into the epithelial membrane of the mouth or the general circulation, through which the virus is allowed to reach the buccal mucosa. Results of cross immunity tests with the virus of foot-and-mouth disease have clearly indicated that parotido-stomatitis is a separate entity.

There are various types of stomatitides of bovines, of viral origin reported from other countries from which the present new affection needs to be differentiated. Infective materials from these were not available for carrying out the cross immunity tests, but in view of their distinctive clinical features as reported in literature such experiments

were really not indicated. Prentice (1913) and Norris and Mettam (1913) described a type of stomatitis in calves occurring at Armagh, a county in Ireland, which was characterized by ulceration in the mouth, more often by "scaly" tongue lesions and no lesions in the feet. Sheep were not susceptible to infective material applied on the scarified buccal mucous membrane. In this disease, on the basis of information received by the authors (Galloway, 1953) no satisfactory controlled experiments have been made in trying to transmit this disease by inoculation with filtrates or suspensions prepared from lesions.

Mason and Neitz (1940) described a viral disease, erosive stomatitis of cattle in South Africa, characterized by ulceration of the lips, dental pad and tongue of calves and other bovines below 3 years of age, and its noninfectivity to sheep and guinea pigs. According to them the disease has much in common with, if not the same as, "Armagh disease." From the description given by Mason and Neitz (1940) we find certain resemblances between erosive stomatitis and the new viral disease investigated by us, such as the nature, their occurrence in young bovines, successful transmission on inoculation of filtrates and suspensions prepared from lesions, and resistance of guinea pigs and sheep to infection. But while these authors described ulceration of the tongue as a constant feature of "erosive stomatitis," no ulcers were seen on the tongue of any of the natural or experimental cases of this disease during our investigation. Further, in erosive stomatitis, pearl's stage has been described prior to ulceration, while no such stage was encountered in this disease. No comparative immunity trials were conducted since all the attempts to obtain the virus of erosive stomatitis and Armagh disease had failed. In view of these differences and the involvement of the parotid salivary gland in this disease, the condition described by the present authors is a distinct disease and bears no relationship with the conditions described by other authors. The disease has no economic importance except when it is likely to be confused with foot-and-mouth disease when its occurrence coincides with or precedes or follows an outbreak of foot-and-mouth disease.

SUMMARY AND CONCLUSIONS

1. An outbreak of a new viral disease of calves which has not so far been recorded in India has been described.

2. This disease has some resemblance to foot-and-mouth disease, although it is different from it.

3. Details of experimental transmission and studies made to differentiate it from other viral diseases have been recorded.

4. In view of the location of the virus and nature of lesions, the disease has been labelled as "parotido-stomatitis."

REFERENCES

Breed, R. S., Murray, E. G. D. and Hitchens, A. P. 1948, Bergey's Manual of Determinative Bacteriology, London, Bailliere, Tindall & Cox.

Galloway, I. A. 1953, Personal communication.

Hagan, W. A. and Bruner, D. W. 1951, The Infectious Diseases of Domestic Animals, London, Bailliere, Tindall & Cox.

Mason, J. H. and Neitz, W. O. 1940, Onderstepoort Journal, **15**: 159–173.

Merchant, I. A. 1950, Veterinary Bacteriology and Virology, The Iowa State College Press.

Norris, J. H. and Mettam, A. E. 1913, Reports of experiments conducted in connection with the suspected outbreak of disease in County Armagh, Appendix B. Report on foot-and-mouth disease in Ireland in the year 1912, Department of Agriculture and Tech. Instruction for Ireland. H.M. Stat. Office (Cod 7103).

Olitsky, P. K., Traum, J. and Schoening, H. W. 1926, Report foot-and-mouth disease Comm. U.S. Dept. Agric. Tech. Bull. 76.

Prentice, D. S. 1913, Report on foot-and mouth disease in Ireland in the year 1912, Dept. of Agric. and Tech. Instruction for Ireland. H.M. Stat Office (Cod 7103).

Traum, J. 1936, J. Am. Vet. Med. Assoc. **88**: 316.

ENHANCEMENT OF CANDIDA INFECTION: DIFFERENTIAL DISTRIBUTION OF RENAL LESIONS IN MICE TREATED WITH AUREOMYCIN

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Since Seligman[1] described the enhancement of candida infection in mice by aureomycin (chlortetracycline), his observations have been confirmed by various observers.[2–4] However, the mechanism of this enhancement remains obscure.

In the course of other studies relating to the epidemiology of candida infection in children,[5] the enhancing effect of aureomycin and other chemotherapeutic agents was reinvestigated in an attempt to learn something of the mechanism of this phenomenon. When the sequence of histological changes characteristic of candida infection in mice was studied, pronounced differences were noted between the development of renal lesions in treated and untreated mice. The purpose of this report is to present the results of experiments done as the consequence of this observation.

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The microphotographs were prepared by Mr. John Carabitses, The Children's Cancer Research Foundation.

1. Seligman, E. 1952, Proc. Soc. Exper. Biol. & Med. **79**: 481–484.
2. Brown, R., Hazen, E. L. and Mason, A. 1953, Science, **117**: 609–610.
3. Cazin, J. and Huppert, M. 1954, Bact. Proc. **54**: 87.
4. Roth, F. J., Jr., Elyar, O. R. and Syverton, J. T. 1954, Bact. Proc. **54**: 87.
5. Winter, W. D., Jr. and Foley, G. E. To be published.

METHODS

The strain of *Candida albicans*[*] used in these experiments was isolated in 1952 from the mouth of a 6-year-old boy with eosinophilic granuloma and was carried by monthly passage on Sabouraud's dextrose agar for about 18 months prior to these studies; it is typical of the species except for very weak acid production from sucrose.[6] All mice were 20 g CFW (Carworth Farms, Webster strain), and of uniform sex in a given experiment; no sex differences were apparent. Mice were inoculated intraperitoneally with 0.2 ml of an 8% suspension of packed cells harvested after 3 to 4 days growth at 37 C on Sabouraud's dextrose agar or in ammonium sulfate broth.[7] The vehicle was 0.9% NaCl, or a solution of the adjuvant, containing the desired inoculum in 0.2 ml. This standard inoculum was found on chamber and plate counts to contain approximately 8×10^6 microoorganisms. When a pharmacologic agent such as aureomycin was used as an adjuvant, the initial dose of the agent was repeated (without microorganisms) on the 2nd and 3rd experimental days. All animals consumed a standard laboratory mouse chow and water ad libitum.

Animals were sacrificed on the 2nd and subsequent days or on the 5th and final day of each experiment. The left kidney was fixed in 10% formalin and sections stained by the periodic acid-Schiff's reagent method.[8] In certain instances the heart blood and peritoneal surfaces were cultured on Sabouraud's dextrose agar.

RESULTS

Mice given the standard inoculum intraperitoneally without adjuvant rarely died, but had positive heart-blood cultures through the 3rd and positive per-

[*] Children's Cancer Research Foundation Collection no. 60112.

6. Winter, W. D., Jr. and Foley, G. E. 1955, J. Infect. Dis. **97**: 227–230.
7. Mager, J. and Aschner, M. 1947, J. Bact. **53**: 283–295.
8. Lillie, R. D. 1947, J. Lab. & Clin. Med. **32**: 910–912.

itoneal cultures through the 5th (last cultured) experimental day. Gross renal lesions were rarely apparent before the 3rd day, appearing then as whitish nodules and increasing in size and number with the passage of time. Microscopically, after 24 hours *Candida* were seen in small clusters scattered throughout the substance of the kidney, with no constant relation to any vascular or tubular structure and unattended by any leucocytic reaction (fig. 1a). The number of microorganisms in these foci had increased by 48 hours and local infiltration with polymorphonuclear and mononuclear leucocytes had appeared (fig. 1b). Within the next day or two, the lesions had become visible macroscopically and had developed the character of miliary pyogenic abscesses (fig. 1c, 2a). By the 5th day the *Candida*, which had been producing hyphae vigorously, usually had begun to diminish in number (fig. 1d), and by the 10th day the lesions had become healing granulomata which were often devoid of microorganisms.

Other mice were inoculated intravenously with approximately 2×10^7 microorganisms in comparable experiments. The character, distribution, and evolution of the lesions developing in these mice were similar to those resulting from intraperitoneal inoculation, except that the glomeruli seemed to be a site of localization more frequently, due perhaps to the unavoidable presence of a few hyphal fragments in the intravenous inoculum. Moreover, the lesions were more advanced, being comparable in 24 hours to those seen 48 hours following intraperitoneal inoculation.

When the standard inoculum was suspended in 5% hog gastric mucin† and injected intraperitoneally, no essential difference from the control infection was observed on gross or microscopic examination. As in mice infected intravenously or intraperitoneally without mucin, the lesions were scattered throughout the substance of the kidney, indicating hematogenous dissemination in each instance.

The administration of aureomycin with the standard inoculum, on the other hand, resulted in a pronounced difference. When given 1.26 mg of aureomycin* intraperitoneally per mouse per day, the mice developed gross lesions which appeared to be similar to those observed in the mucin-treated and untreated controls. However, on section these lesions proved to be superficial: the entire peritonealized surface of the kidney was invaded by diffuse or focalized hyphal growth (fig. 2b), and there was a considerable invasion of the perirenal fat, which was not observed in the mucin- and untreated controls. Cellular reaction to the invading microorganisms was of the same character and appeared at the same time as in either group of control mice. Lesions deep in the substance of the kidney were by contrast rare and did not appear before the 4th or 5th day. These lesions clearly represented a secondary and rather limited hematogenous spread. Cellular reaction was delayed for 24 hours after appearance of the microorganisms, as was observed in the typical early lesions.

The sodium glycinate contained in the aureomycin preparations used had no relation to the results obtained, since similar therapy with the same dose of pure, crystalline aureomycin produced the same histological pattern, while therapy with sodium glycinate in the dosage contained in the buffered aureomycin preparations resulted in lesions indistinguishable from those observed

† Wilson Granular Mucin, type 1701-W.

* 2 mg of Lederle aureomycin hydrochloride crystalline intravenous, containing 37% sodium glycinate as buffer.

in mucin- and untreated control mice. Substitution of terramycin (oxytetracycline) for aureomycin gave qualitatively similar effects. However, tetracycline gave somewhat irregular results with various combinations of deep and superficial lesions in different mice.

A saline suspension of cortisone acetate† in doses of 5 mg per mouse per day intraperitoneally on the first 3 days after inoculation gave a distribution of lesions identical with that of aureomycin (fig. 2c).

The death rate was low (under 5% in 3 days) among the untreated and cortisone-treated mice (table 1). Unlike observations reported by others,[9,10] the administration of hog gastric mucin in combination with the inoculums used had no significant effect upon the mortality resulting from infection with this strain of *C. albicans*. A 4-fold dilution of the standard inoculum injected intraperitoneally failed to produce gross lesions by the 5th experimental day in 90% of the mice studied. Again, the addition of hog gastric mucin to such diluted inoculums had no effect either upon the mortality rate or the frequency of lesions at autopsy.

However, a 4-fold dilution of the standard inoculum produced the characteristic superficial lesions in the majority of the mice receiving aureomycin therapy, although most of the mice survived to the 5th day following infection with these diluted inoculums (table 1). Cortisone therapy was even more effective with respect to this enhancing effect, a 100-fold dilution of the standard inoculum producing the characteristic superficial lesions in most of the mice despite the lack of mortality with such infections (table 1).

TABLE 1.—*Effect of aureomycin and cortisone on mortality rate and pathogenicity in mice inoculated with* C. albicans.

Inoculum	Treatment		
	None	Aureomycin*	Cortisone†
	Mortality: Cumulative through 3rd day		
Standard‡	6/123§	23/35	0/10
1:2	0/40	3/20	
1:4	0/40	0/20	
1:10	0/10	0/10	0/10
1:100	0/10	0/10	0/10
	Gross renal lesions: 5th day		
Standard	87/105	9/9	10/10
1:2	27/35	8/12	
1:4	5/35	12/15	
1:10	0/9	8/10	9/10
1:16		7/10	
1:30		1/10	8/10
1:100	0/10	1/10	8/10

* 1.26 mg/mouse/day intraperitoneally, first 3 days.
† 5 mg/mouse/day intraperitoneally, first 3 days.
‡ 0.2 ml of 8% suspension of packed cells, or dilution thereof.
§ Animals affected (numerator) over total animals (denominator).

DISCUSSION

The initial experiments using intraperitoneal inoculation indicate that the spread of *Candida* from the peritoneal cavity normally occurs by hematogenous dissemination. The lesions produced are essentially identical in character and distribution to those produced by direct intravenous inoculation, except that the latter are approximately 24 hours more advanced.

It is apparent from subsequent experiments that the intraperitoneal administration of aureomycin or cortisone to mice inoculated with *C. albicans* profoundly alters the usual course of events. The scattered and unquestionably hematogenous renal lesions appear late and infrequently in treated mice, while in their stead there is vigorous invasion of the peritonealized portion of the renal cortex. On the one hand, aureomycin seems to exert a beneficial effect by delaying the appearance of the deep and scattered renal abscesses, despite the fact that cultures demonstrate the presence of *Candida* in the peripheral

† Merck and Co., Inc., Sharp and Dohme, Inc.

9. Strauss, R. E. and Kligman, A. M. 1951, J. Infect. Dis. **88**: 151–155.
10. Salvin, S. B., Cory, J. C. and Berg, M. K. 1952, J. Infect. Dis. **90**: 177–182.

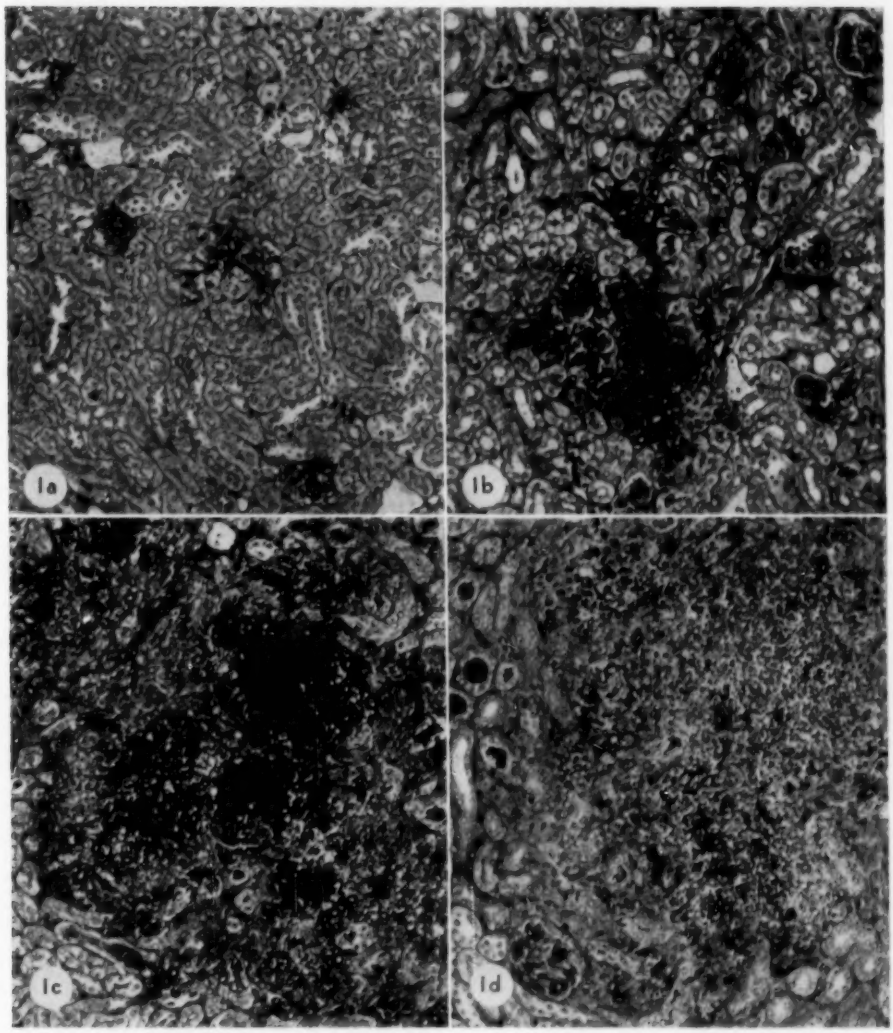
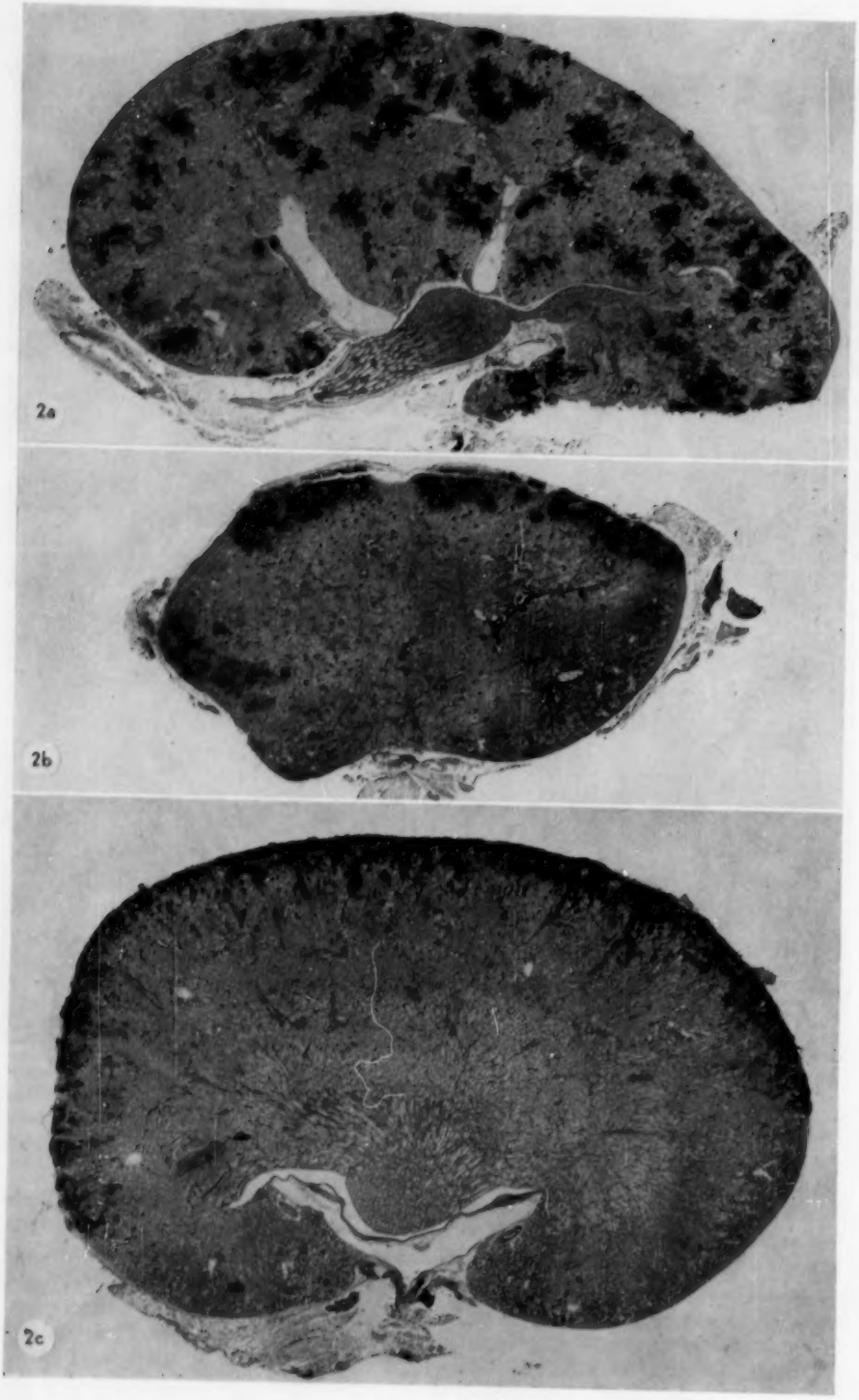


FIG. 1.—Mouse kidney (a) 24 hours after intraperitoneal inoculation of *C. albicans*, showing early development of hyphae with minimal tissue necrosis and no leucocytic reaction; (b) 48 hours: lesion advancing with initial cellular reaction; (c) 72 hours: well developed abscess; (d) 120 hours: note diminution of candida elements as lesion begins to heal. PAS stain, ×150.

blood. Seligman[11] noted that aureomycin therapy reduced the mortality rate among mice injected intravenously with *Candida*. On the other hand, a simultaneous and presumably deleterious influence is exerted, fostering direct invasion through the renal capsule. At the same time, the mortality rate increases, especially during the first 72 hours fol-

11. Seligman, E. 1953, Proc. Soc. Exper. Biol. & Med. **83**: 778–781.


2a
2b
2c

lowing infection. The cause of death in treated mice is obscure; the number and anatomical distribution of the lesions does not provide sufficient explanation. Untreated control mice survive the loss of larger amounts of renal tissue, and in either group, other tissues and organs are essentially unaffected. The dose of aureomycin used is nontoxic. In addition to the increased mortality, pathogenicity is definitely increased, as evidenced by the effectiveness of subinfective inoculums when combined with aureomycin. Seligman[11] stated that the administration of the antibiotic by the intraperitoneal route alone was effective for enhancement, which suggests that a local irritative effect might play a role. Since the dose used was nontoxic, it is difficult to understand, if the mechanism of action of aureomycin were so simple, why the number of deep parenchymal lesions is reduced by therapy and why there is no increase in the infrequent lesions observed elsewhere on peritoneal surfaces.

That cortisone enhances the pathogenicity of several microbiologic agents is a well established principle.[12] However, that its effect would be so directly comparable to that of aureomycin in candida infection in mice was not anticipated. Since the activity of cortisone in infectious disease generally is considered to be the result of altered host reactivity,[12] one is tempted to infer that a similar relationship may obtain in the case of aureomycin. While the information presented here is as yet too meager to establish such a conclusion, the data suggest that the "enhancing" effect of aureomycin on candida infection represents a complex biological phenomenon in which host response is a significant factor, rather than a simple stimulatory effect exerted upon the fungus. Whether or not these observations on candida infection in mice are related to the changes in renal physiology induced in man by oxytetracycline[13–15] and cortisone[16] can be determined only by additional studies now in progress.

The discrepancy between mortality and the presence of significant pathology at autopsy as observed in these experiments re-emphasizes an observation made some time ago, viz., that mortality is not necessarily the best end point for use in the study of experimental infection,[17] particularly in the evaluation of an enhancing agent. Indeed, in the case of experimental tuberculosis in the guinea pig,[17] quantitation of the extent of pathologic change at autopsy revealed marked differences among control and hormone-treated animals even in the absence of marked differences in mortality.

SUMMARY

The spread of *Candida albicans* following the intraperitoneal injection of standard inoculums in CFW mice was

12. Kass, E. H. and Finland, M. 1953, Ann. Rev. Microbiol. **7**: 361–388.
13. Smith, O. W. and Roby, C. C. 1955, J. Clin. Investigation. In press.
14. Gabuzda, G. F., Jr., Gocke, T. M. and Love, B. D., Jr. 1953, J. Clin. Investigation, **32**: 570.
15. Gabuzda, G. F., Jr., Jackson, G. G. and Grisby, M. E. 1952, **31**: 631.
16. Ingbar, S. H., Kass, E. H., Burnett, C. H., Relman, A. S., Burrows, B. A. and Sisson, J. H. 1951, J. Lab. & Clin. Med. **38**: 533–541.
17. Aycock, W. L. and Foley, G. E. 1945, J. Clin. Endocrinol. **5**: 337–344.

FIG. 2.—(a) Mouse kidney 4 days after intraperitoneal inoculation of *C. albicans*. Note scattered foci. PAS stain, ×13. (b) Same inoculum but treated with aureomycin hydrochloride, 1.26 mg intraperitoneally on experimental days 1, 2, and 3. Note peripheral distribution of lesions. Punctate objects are normal glomeruli. ×13. (c) Same inoculum, treated with cortisone acetate, 5 mg intraperitoneally on experimental days 1, 2, and 3. Lesions are entirely peripheral. ×17.

found to be by direct hematogenous dissemination. The major pathology was confined to the renal tissues and consisted of scattered miliary abscesses deep in the substance of the kidney.

The administration of hog gastric mucin had no significant effect either upon mortality or the character of renal lesions. Similar results were observed in mice infected intravenously, except that the renal lesions developed more rapidly.

The administration of aureomycin, terramycin or tetracycline to mice infected with standardized inoculums not only increased mortality early in the course of infection, but resulted in a massive invasion of the renal cortex from the peritonealized surfaces of the kidney, in contrast to the deep-seated renal lesions observed in mucin- and untreated mice. Cortisone acetate therapy resulted in lesions identical with those observed in mice treated with aureomycin.

Aureomycin or cortisone acetate therapy increased the pathogenicity of *C. albicans*, as evidenced by development of the characteristic renal lesions following injection of inoculums which were ineffective in untreated mice. This enhancement of pathogenicity appeared to be associated with decreased hematogenous dissemination.

ANTIBODY AND GAMMA GLOBULIN FORMATION IN VITRO IN HEMOPOIETIC ORGANS

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In previous publications (Keuning and van der Slikke, 1950; Thorbecke and Keuning, 1953) the formation of antibodies in tissue cultures of the spleen, bone marrow, and lymph nodes after subcutaneous and intravenous immunization has been described. In all these experiments, the highest amount of antibody was found to be formed in the red pulp of the spleen. Little antibody formation was found in the white pulp of the spleen and no significant production of antibody in the liver and thymus. An apparent correlation existed between the occurrence of young plasma cells and the formation of antibodies in vitro in the organs investigated.

It is well known that both in the human and in many animal species antibodies are largely bound to or, more probably, identical with circulating proteins belonging to the γ globulin fraction of the serum proteins (Enders, 1944; Haurowitz, 1953; van der Scheer et al, 1941a and b, 1942). Only a few antibodies have been found in the β globulin fraction or in a fraction intermediate between the β and the γ globulins.

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Further, the authors wish to acknowledge the valuable help of Mr. Heikens, Department of Anatomy, in his drawing of plates 1 and 2a.

Comparatively little is known concerning the formation of these globulins and their relationship with the immune substances comprised by them. Regarding the organs responsible for plasma protein synthesis Miller et al (1951, 1954a and b) demonstrated in rats that all albumin and 80% of the globulins were formed by the liver, these globulins being mainly α and β globulins. A small part of the β and all of the γ globulin seemed to be formed elsewhere in the body.

It has been suggested that γ globulin is produced by plasma cells. The main point in support of this hypothesis is the relationship that has been established in the human being between high γ globulin levels in the blood plasma and the proliferation of plasma cells in several organs in a number of diseases, mostly infectious or allergic in origin (Bing, 1940; Hayhoe and Smith, 1951; Klein and Block, 1953; Müting, 1951; Schlienger, 1945). There is little doubt that young plasma cells form γ globulins so far as the latter are, in fact, immune globulins.

However, the relationships between normal γ globulins and immune globulins and the exact mechanism by which antibodies are formed have remained obscure, although several hypotheses have been put forth. Antibodies might be formed by (1) a conversion of normal γ globulins into antibodies (Loiseleur, 1951); (2) a deviation of normal γ globulin synthesis under the influence of an assimilated antigen; (3) the independent formation out of amino acids of entirely new proteins having the

character of γ globulins (Green and Anker, 1954). An elucidation of the relationship between "normal" and "immune" γ globulin would perhaps lead to a better understanding of the way in which antigens induce the formation of antibodies, i.e., of new, specifically reacting protein.

In this paper first a more detailed description will be given of the histology of the plasmacellular reaction, particularly in the spleen, during antibody formation. Furthermore, with regard to the formation of antibodies in spleen, bone marrow and lymph node cultures, we investigated whether this increase of antibodies in vitro could also be demonstrated as an increase of γ globulins as such. Finally, this paper will present the results of experiments concerning γ globulin formation, with a discussion as to how these results can be interpreted in the light of some data known from the literature.

MATERIALS AND METHODS

Throughout the experiments young adult rabbits were used, a number of which were immunized either with parathypoid B vaccine (H antigen)* or with horse serum.* Parathyphoid immunization was usually done by 3 weekly intravenous injections (tables 1 and 2). Horse serum immunization was performed by 4 daily intravenous injections of increasing amounts (0.15, 0.3, 0.45, 0.6 ml) each week during a period of 3 or 4 weeks (table 3).

Three days after the last antigen injection the animals were killed by bleeding, following the administration of 50 mg of heparin intravenously. The organs to be cultured were removed aseptically and a number of minute pieces were prepared from each organ. Each lot of tissue fragments was then divided by inspection into two equal parts. In preliminary experiments it was found that the errors of division made in this manner were less than the errors inherent to the titration methods. One-half of the tissue fragments was used to prepare an "extract"; it was immediately macerated with quartz meal, frozen and thawed three times to lyse the cells, and stored with 2 ml of culture medium in the refrigerator. The tissue fragments of the other half, the "culture," were transferred into roller tubes and cultured with 2 ml of culture medium at 37 C for a period of about 28 hours. The culture medium usually consisted of a mixture of tyrode solution, rabbit serum and amparon.† If lymphoid parts of the intestine were cultured (appendix or patches of Peyer), 1 mg of streptomycin was added to each culture. Whenever γ globulin production was to be demonstrated, calf serum was used instead of rabbit serum. After the culture period the cultures were also macerated in their own culture fluid and three times frozen and thawed. Antibody and γ globulin titrations were then performed simultaneously on the fluids of corresponding "extracts" and "cultures." If a higher content of antibodies and/or γ globulin was found in a culture than in the corresponding extract, it could then be concluded that this was the result of a formation in vitro during the culture period (Fagraeus, 1948; Keuning and van der Slikke, 1950; Mountain, 1955).

Titers and production indexes.—Anti-paratyphoid H agglutinin titers were determined with the aid of H antigen, prepared by formol treatment of bacterial cells; the double dilution technique was used, the antibody containing fluids being diluted. Anti-horse serum precipitin titers were determined with the surface precipitation method, in which the antibody was diluted and tested with constant amounts of undiluted horse serum. The interfacial precipitin reaction was also used for the determination of γ globulin titers, in this case the antigen containing fluids being diluted and tested with constant amounts of an undiluted rabbit γ globulin precipitating serum. The production indexes were calculated from these titers as follows: If an extract titer was read to be 1:4 and the corresponding culture titer 1:32, the production was calculated to be $(32-4)/4=7\times$. As the quantities of tissue used from different organs were not always the same, these production indexes are of much more importance for a direct comparison of the cultured organs than the titer values themselves.

Preparation of the rabbit γ globulin precipitating serum.—Rabbit γ globulin was prepared from rabbit serum with sodium sulfate precipitation, according to Majoor (1947). An alum precipitate of this γ globulin served to immunize a goat (Proom, 1943; Vink, 1947). The antiserum obtained in this way gave a surface precipitation with a 1:1500 dilution of a 2% rabbit γ globulin solution.

* The antibodies against these antigens mainly belong to the γ globulin fraction of the serum.

† Amparon is a 5% solution of a hydrolysate of casein prepared with papain (N. V. Organon, Oss.)

Serum protein determinations.—Total protein determinations were performed according to Weichselbaum (1946). Serum protein fractions were determined either with the Tiselius method or with paper electrophoresis (modification of Macheboeuf's method (1953), staining with a bromophenol blue method adapted for quantitative work (Keuning, 1954). In the calculations of the paper electrophoresis, proteins that had stayed on the line of departure together with hemoglobin, if present, were regarded as one fraction and omitted from the tables. No correction factor for globulin values has been applied in these experiments. Plasmapheresis (Wuhrmann, 1947) was performed on some animals. The amounts of blood replaced by a suspension of the rabbit's own erythrocytes through a number of heart punctures over a certain period are included in table 6. Antibiotics were given to prevent intercurrent infections (table 6).

Histological methods.—Tissues were fixed in a mixture of Zenker's fluid with formalin and/or with formalin and 2% trichloroacetic acid (90:5:5). Paraffin sections were stained with methylgreen pyronin (Koch's modification (1913) of the Unna Pappenheim procedure). In accordance with Brachet (1953) short fixation periods were found to give better results with this staining method.

RESULTS

Histological observations on the spleen during antibody formation.—In the spleen, the formation of plasma cell aggregates is very conspicuous during immunization. The primary origin of these cells was not clear in our slides. We failed to find definite support for the hypothesis that they are derived from reticulum cells, as stated by Fagraeus (1948) and Marshall and White (1950). Some 2 to 3 days after the administration of an antigen considerable numbers of large actively proliferating blast cells, the so-called "plasmablasts," were always found in the red pulp. Most of them were lying closely together in groups, the characteristic aggregates. These plasmablasts are rounded cells with large pale nuclei and prominent nucleoli and a narrow zone of strongly basophilic (pyroninophilic) cytoplasm.

In the course of the following two days these cells became somewhat smaller and still more basophilic; their nuclei became darker through condensation of chromatin and took an eccentric position in the cell; nucleoli were still clearly visible. In this stage a clear juxtanuclear zone was usually observed in the cytoplasm. These cells may be called immature plasma cells.

After another two days most of these cells had the typical morphology of mature, Marschalko-type plasma cells: a small, round nucleus, lying eccentricly in the cell, heavily staining chromatin clumps, no visible nucleolus and a strongly basophilic cytoplasm with a characteristic juxtanuclear clear zone.

These aggregates of plasma cells, particularly the younger forms, were found to have a very definite localization. They were always found bordering the white pulp lymphoid tissue, surrounding the smallest arterioles and arterial capillaries emerging from the white pulp (pl. 1, 2a and 2b). Even plasma cell aggregates that were apparently lying free in the red pulp (pl. 3) could always be found to have this same relationship with smallest vessels and white pulp, when examined in serial sections.

More difficult to classify were cells that looked exactly like the "plasmablasts" and were located more or less diffusely throughout the lymphoid tissue of the white pulp around the arterioles, with perhaps a preference for localization in the border of this lymphoid tissue. The relationship of these cells, possibly large lymphoid stem cells, to the proliferation of the plasma cells in the red pulp was difficult to establish, particularly in view of the changing structure of the lymphoid sheaths. It was obvious, however, that plasma cells did never occur in this localization, i.e., inside the white pulp. Neither did we find any support for the statement of

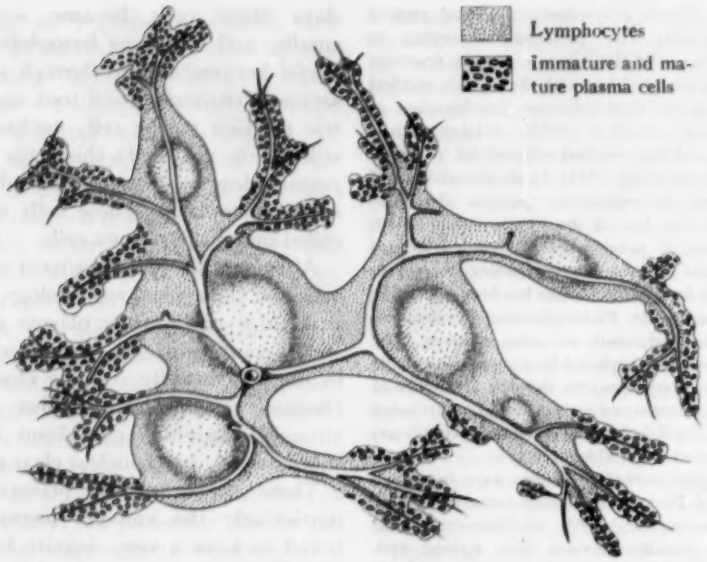


FIG. 1.—Spleen of rabbit during immunization showing relationship between white pulp and plasma cell aggregates. Graphic reconstruction (projection) from serial sections.

Moeschlin (1951) that there should be two different kinds of plasma cells in the the spleen.

Furthermore, a hypertrophy of the white pulp and the development of typical "reaction" centers in the Malpighian corpuscles was always observed after the mode of immunization used in this investigation. These active "reaction" centers showed many small basophilic lymphoid stem cells, the medium-sized lymphocytes of Maximow, with many mitoses and signs of cell disintegration and phagocytosis ("starry sky" appearance).

Antibody formation and plasma cells in the liver.—In former experiments no antibody production was found in the liver after intravenous immunization with paratyphoid vaccine (Thorbecke and Keuning, 1953). This, however, is in contradiction to the findings of Ranney and London (1951). The possibility was considered that immunization after splenectomy or hyperimmunization might stimulate the liver to the production of antibodies. Some additional experiments were therefore performed on animals that had been splenectomized one-half to one year previously. The results are summarized in table 1.

It was found that after immunization with paratyphoid B vaccine in splenectomized rabbits the livers sometimes did form a small amount of antibodies in vitro. The same was sometimes found after immunization with horse serum in normal animals. Upon microscopic examination these livers of immunized splenectomized rabbits showed a distinct histological reaction, more pronounced than the livers of immunized intact animals did. These changes consisted of a reaction of the periportal connective tissue in which aggregations of lymphocytes together with mature and immature plasma cells were observed (pl. 4). The liver parenchyme itself never showed any changes.

TABLE 1.—*The formation of paratyphoid B agglutinin in vitro after intravenous immunization in splenectomized rabbits.*

Rabbit no.	Bone marrow	Liver	Mesenteric lymph node	Thymus	Mode of immunization	Culture medium
246						
E titer	1:128	1:32			3 weekly "recall" injections* of 0.3, 0.4 and 0.5 ml vaccine	Tyrode solution, calf serum 5%, amparon 5%
C titer	1:512	1:32				
Production	3×	0×				
244						
E titer	1:128	1:128			3 weekly recall injections*	Tyrode solution, calf serum 5%, amparon 5%
C titer	1:512	1:256				
Production	3×	1×				
229						
E titer	1:32	1:8		1:4	2 weekly recall injections*	Tyrode solution, calf serum 5%, amparon 5%
C titer	1:48	1:8		1:2		
Production	½%	0×		0×		
230						
E titer	1:16	1:8	1:16	1:1½	2 weekly recall injections*	Tyrode solution, rabbit serum 10%, amparon 10%
C titer	1:96	1:8	1:32	1:1		
Production	5×	0×	1×	0×		
228						
E titer	1:8	1:8	1:8	1:8	2 weekly recall injections*	Tyrode solution, rabbit serum 10%, amparon 10%
C titer	1:32	1:16	1:12	1:8		
Production	3×	1×	½×	0×		
233						
E titer	1:64	1:64			3 weekly injections	Tyrode solution, calf serum 5%, amparon 5%
C titer	1:512	1:128				
Production	7×	1×				
280						
E titer	1:256	1:64	1:24		6 weekly injections	Tyrode solution, calf serum 5%, amparon 5%
C titer	1:512	1:64	1:32			
Production	1×	0×	½×			

* These animals had been immunized a considerable time before these experiments were performed; therefore the antigen injections are called "recall" injections.

The parallelism between antibody and γ globulin formation in vitro.—Tables 2, 3 and 4 give the results of experiments in which both antibody and γ globulin formation were determined in various organs in vitro after intravenous immunization. Tables 2 and 4 give the results after paratyphoid B immunization in normal and splenectomized rabbits, respectively; table 3 summarizes

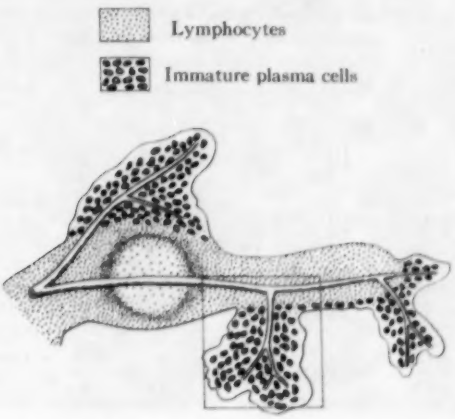


FIG. 2a.—Spleen of rabbit during immunization: white pulp and plasma cell aggregates. Graphic reconstruction from serial sections.

TABLE 2.—*Antibody (A) and γ globulin (P) production in rabbits after intravenous paratyphoid immunization.*

Rabbit no.	Spleen		Bone marrow		Liver		Mesenteric lymph node		Thymus		Immunization	Culture medium
	A	P	A	P	A	P	A	P	A	P		
273											3 weekly injections of 0.3, 0.4, and 0.5 ml vaccine	Tyrode, calf serum 5%, amparon 5%
E titer	1:128	1:16	1:96	1:16	1:24	1:8						
C titer	1:512	1:32	1:256	1:32	1:32	1:12						
Production	3×	1×	1½×	1×	0×	½%						
265											3 weekly injections	Tyrode, calf serum 5%, amparon 5%
E titer	1:64	1:16	1:16	1:4	1:8	<1:1			1:2	<1:1		
C titer	1:512	1:58	1:192	1:16	1:8	<1:1			1:2	<1:1		
Production	7×	3×	11×	3×	0×				0×			
302											3 weekly injections	Tyrode, calf serum 4%, amparon 4%
E titer	1:256	1:4	1:128	1:32	1:64	1:16	1:128	1:8				
C titer	1:3072	1:12	1:512	1:32	1:64	1:16	1:256	1:16				
Production	11×	2×	3×	0×	0×	0×	1×	1×				
286											5 weekly injections	Tyrode, calf serum 5%, amparon 5%
E titer	1:32	1:1	1:24	1:4	1:128	1:8						
C titer	1:512	1:6	1:128	1:8	1:128	1:6						
Production	15×	5×	5×	1×	0×	0×						
	Appendix											
282											3 weekly injections	Tyrode, calf serum 4%, amparon 4%
E titer	1:96	1:4	1:96	1:8	1:32	1:8	1:24	1:4	1:12	1:2		
C titer	1:256	1:6	1:768	1:16	1:48	1:8	1:48	1:4	1:8	1:2		
Production	2×	½×	7×	1×	½×	0×	1×	0×	0×	0×		
	Liver								Peyer's patches			
278											3 weekly injections	Tyrode, calf serum 5%, amparon 5%
E titer	1:128	1:4	1:128	1:4	1:64	1:8			1:64	1:8		
C titer	1:512	1:8	1:392	1:8	1:64	1:8			1:64	1:8		
Production	3×	1×	2×	1×	0×	0×			0×	0×		

the titers and production indexes after horse serum immunization.

It was found that antibody formation in a culture was usually accompanied by an increase in γ globulin, while in cases without antibody formation in vitro also no increase in γ globulin was found during the culture period.

The data of table 3 show a ratio between antibody and γ globulin titers different from those of table 2. This is explained by several circumstances, but the most important factor is probably a technical one. While γ globulin titers,

TABLE 3.—*Antibody (A) and γ globulin (P) production in rabbits after intravenous horse serum immunization.*

Rabbit no.	Spleen		Bone marrow		Liver		Immunization	Culture medium
	A	P	A	P	A	P		
258							4 weeks	Tyrode, calf serum 5%, amparon 10%
E titer	1:4	1:4	1:2	1:4	1:2	1:2		
C titer	1:32	1:96	1:4	1:24	1:12	1:8		
Production	7×	23×	1×	5×	5×	3×		
259							3 weeks	Tyrode, calf serum 5%, amparon 5%
E titer	1:1	1:4	1:1	1:2	<1:1	1:1		
C titer	1:5	1:32	1:2	1:4	<1:1	1:1		
Production	4×	7×	1×	1×		0×		
2791							4 weeks	Tyrode, calf serum 5%, amparon 5%
E titer	1:12	1:16	1:3	1:16	1:4	1:8		
C titer	1:16	1:32	1:2	1:8	1:6	1:16		
Production	0×	1×	0×	0×	½×	1×		
260							4 weeks	Tyrode, calf serum 5%, amparon 5%
E titer	1:2	1:12	<1:1	1:6	1:2	1:16		
C titer	1:4	1:64	<1:1	1:8	1:2	1:16		
Production	1×	4×		0×	0×	0×		
289							6 weeks	Tyrode, calf serum 5%, amparon 5%
E titer	1:8	1:8	1:3	1:8	1:8	1:8		
C titer	1:6	1:8	1:4	1:12	1:8	1:8		
Production	0×	0×	0×	½×	0×	0×		

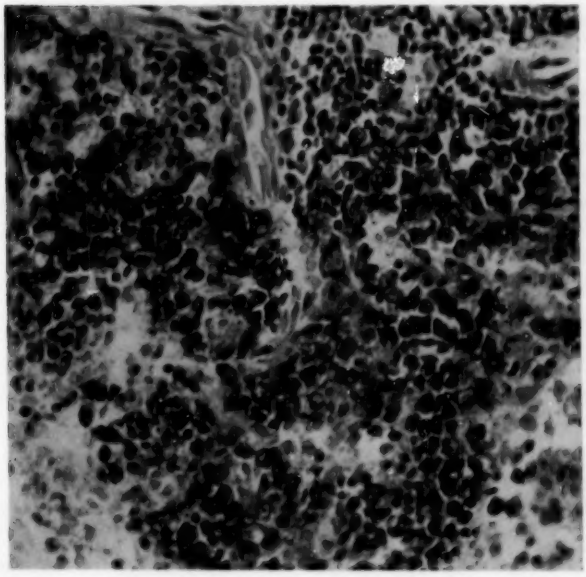


FIG. 2b.—Micrograph corresponding to rectangle from figure 2a. Aggregate of plasmablasts and immature plasma cells surrounding arteriole emerging from white pulp.

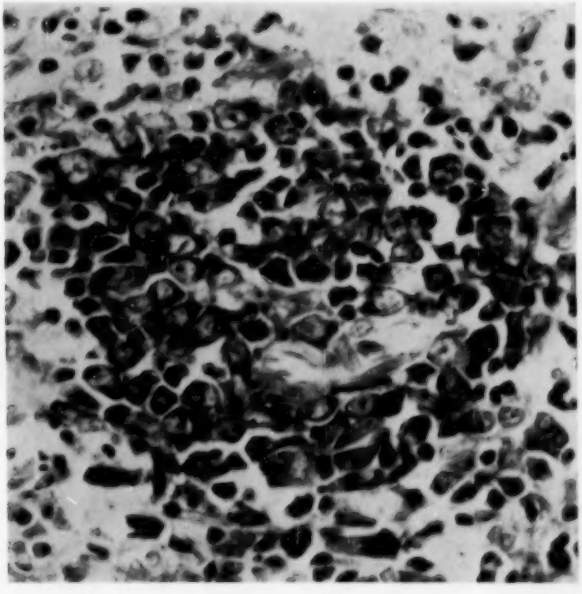
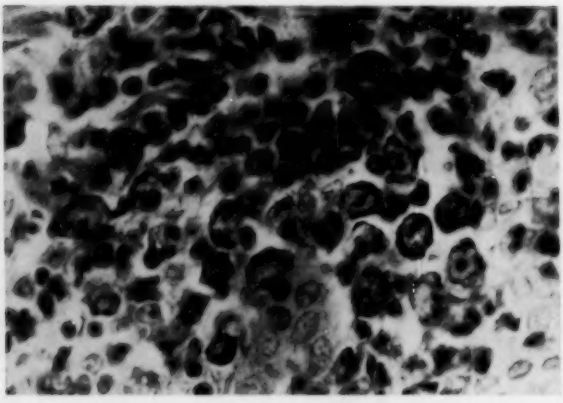


FIG. 3.—"Isolated" aggregate of plasmablasts and immature plasma cells, surrounding small arteriole. Serial sections showed continuity with white pulp.



↑
Bile duct

FIG. 4.—Plasmacellular reaction with plasmablasts in periportal space of liver. From a splenectomized and subsequently immunized rabbit.

as described above, were determined with the interfacial precipitation end point method, in which serial antigen dilutions were used, the anti-horse serum precipitation was done with serial antibody dilutions—necessary because of the limited amounts of extract and culture fluids, but less sensitive. The agglutination method used to determine paratyphoid antibodies, on the contrary, is very sensitive. One should therefore not make the mistake of directly comparing the titers found by different methods, but should use the results of each method separately as a measure of increase of the titrated substance in vitro during the culture period.

To make sure that the γ globulin for-

TABLE 4.—*Antibody (A) and γ globulin (P) production after intravenous paratyphoid immunization in splenectomized animals.*

Rabbit no.	Bone marrow		Liver		Mode of immunization	Culture medium
	A	P	A	P		
246						
E titer	1:128	1:4	1:32	1:2	See table 1	Tyrode, calf serum 5%, amparon 5%
C titer	1:512	1:12	1:32	1:2		
Production	3×	2×	0×	0×		
244						
E titer	1:128	1:16	1:128	1:32	See table 1	Tyrode, calf serum 5%, amparon 5%
C titer	1:512	1:64	1:256	1:48		
Production	3×	3×	1×	½×		
229*						
E titer	1:32	1:16	1:8	<1:1	See table 1	Tyrode, calf serum 5%, amparon 5%
C titer	1:48	1:24	1:8	<1:1		
Production	½×	½×	0×			
233						
E titer	1:64	1:3	1:64	1:1	See table 1	Tyrode, calf serum 5%, amparon 5%
C titer	1:512	1:16	1:128	1:4		
Production	7×	4×	1×	3×		
280						
E titer	1:256	1:2	1:64	1:1	6 weekly injections of 0.5 ml paratyphoid vaccine	Tyrode, calf serum 5%, amparon 5%
C titer	1:512	1:3	1:64	1:1		
Production	1×	½×	0×	0×		

* The thymus of rabbit 229 was also cultured. It showed no antibody and no γ globulin formation.

mation found in these cultures, parallel to the formation of antibodies, was an actual synthesis of immune substances, similar organs were taken from normal (nonimmunized) animals and cultured (table 5). In the organs of these normal animals a significant increase of γ globulin during the culture period was never established.

We tried, therefore, in normal animals to stimulate γ globulin production to such levels as to be demonstrable with our methods of culturing. Loss of plasma protein by plasmapheresis would seem the most logical way of stimulation. In these plasmapheresis experiments the "in vitro" γ globulin production, the regeneration rates of serum protein of the intact animal, and, histologically, a possible increase of immature plasma cells were investigated.

The results of these tissue culture experiments and of the regeneration studies with electrophoresis are given in table 6 and 7, respectively. Table 6 shows that, in spite of the appreciable loss of plasma proteins during plasmapheresis, no significant production of γ

TABLE 5.—*γ globulin production in vitro in organs of nonimmunized rabbits.**

Rabbit no.	Spleen	Bone marrow	Liver	Thymus	Peyer's patch	Mesenteric lymph node	Appendix
226							
E titer		1:4		1:16	1:8		1:2
C titer		1:8		1:6	1:8		1:2
Production		1×		0×	0×		0×
304							
E titer	1:8	1:16		1:32	1:8		
C titer	1:8	1:8		1:32	1:8		
Production	0×	0×		0×	0×		
307							
E titer	1:12	1:32			1:16		
C titer	1:12	1:96			1:16		
Production	0×	2×			0×		
236							
E titer	1:4				1:1	1:1	
C titer	1:4				1:1½	1:1	
Production	0×				½×	0×	
274							
E titer	1:6	1:4	1:16		1:4		
C titer	1:6	1:4	1:16		1:4		
Production	0×	0×	0×		0×		
196							
E titer	1:8		1:4		1:8		
C titer	1:8		1:4		1:8		
Production	0×		0×		0×		
261							
E titer	1:6	1:8			11:6		
C titer	1:6	1:8			11:6		
Production	0×	0×			0×		
173							
E titer	1:12				1:8		
C titer	1:8				1:8		
Production	0×				0×		
174							
E titer	1:16				1:6		
C titer	1:16				1:8		
Production	0×				0×		
279†							
E titer	1:8						
C titer	1:8						
Production	0×						
280†							
E titer	1:4						
C titer	1:4						
Production	0×						

* Culture medium: tyrode, calf serum 5%, amparon 5%.
† Spleen removed surgically.

TABLE 6.—*γ globulin production in vitro in rabbits after plasmapheresis.*

Rabbit no.	Spleen	Bone marrow	Mesenteric lymph node	Amount of blood taken	Daily dose of antibiotics	Culture medium
315				About 50 ml every other day during 2 weeks	Depocillin 15000 E.i.m., aureomycin 250 mg per os	Tyrode, calf serum 8%, amparon 8%
E titer	1:2	1:3	1:2			
C titer	1:2	1:2	1:2			
Production	0×	0×	0×			
318				About 50 ml every other day during 2 weeks	Depocillin 15000 E.i.m.	Tyrode, calf serum 10%, amparon 10%
E titer	1:2	1:2	1:1			
C titer	1:2	1:2	1:1½			
Production	0×	0×	½×			
262				About 12 ml every day during 1 week	Streptomycin 60 mg i.m.	Tyrode, calf serum 4%, amparon 4%
E titer	1:3	1:1				
C titer	1:2	1:1				
Production	0×	0×				
263				About 12 ml every day during 1 week	Streptomycin 60 mg i.m.	Tyrode, calf serum 4%, amperon 4%
E titer	1:2	1:2				
C titer	1:2	1.2				
Production	0×	0×				
268				About 25 ml every other day during 2 weeks	Streptomycin 50 mg i.m., depocillin 20000 E.i.m., sulfenthidin 200 mg per os	Tyrode, calf serum 5%, amparon 5%
E titer	1:16	1:4				
C titer	1:16	1:4				
Production	0×	0×				
269				About 25 ml every other day during 3½ weeks	Streptomycin 50 mg i.m. penicillin 100,000 E.i.m., sulfenthidin 200 mg per os	Tyrode, calf serum 5%, amparon 7½%
E titer	1:4	1:2				
C titer	1:4	1:3				
Production	0×	½×				

* The liver of rabbit 318 was also cultured; it showed no γ globulin production.

globulin was found in the cultured organs of these animals. Furthermore, in the regeneration of the plasma proteins, there seemed to be a difference between the α and β globulins, which increased after plasmapheresis, and the albumin and γ globulin that decreased.

Of the latter two, the decrease of the γ globulin seemed the most pronounced and sustained (table 7). In dogs after plasmapheresis, Mushett et al (1949) found that albumin, α_1, α_2 and β_2 globulin stayed about at the same level, while α_2 and β_1 globulin increased and γ globulin decreased. Thus it seems that if we can assume the reserves in the body to be proportionally the same for all serum proteins, γ globulin regenerates slower than the other serum proteins. Also it is evident that, under the con-

TABLE 7.—*Serum proteins before and immediately after plasmapheresis.**

Rabbit no.	Percent total protein	Percent albumin	Percent globulins		
			$\alpha_1+\alpha_2$	β	γ
315 (paper E)					
Before	6.7	67.8	9.5	10.2	7.4
After	4.7	56.7	15.4	14.3	5.4
318 (paper E)					
Before	6.1	57.8	16.2	14.1	7.8
After	4.1	47.3	22.9	16.3	4
269 (free E)					
Before		69.7	7.7	13.3	9.2
After	6	64.2	8.7	21.2	6
268 (free E)					
Before		52.1	10.7	20.2	17
After	5.9	49.1	10.9	28.5	11.5
262 (free E)					
Before	5.1	70.5	8.5	13.4	7.6
After	4.9	58.8	20	16.8	4.7
263 (free E)					
Before		66.4	9	17.9	6.7
After		55.2	11.9	25.1	7.8

* See table 6.

ditions of our experiments, the administration of an antigen is a better stimulant for the production of γ globulin than the loss of plasma proteins.

Histologically, the observation of Fagraeus (1948) was confirmed, i.e., that the loss of plasma proteins did not stimulate the production of plasma cells, while the injections of antigens do so, as was mentioned before.

DISCUSSION

Plasmacellular reaction and antibody formation.—Abundant literature is available concerning the rôle of plasma cells, especially the immature ones, in the formation of antibodies and the histogenesis of the plasmacellular reaction induced by administration of antigen.

Apparently the transportation of antigen into lymphoid tissue, or its penetration into connective tissue, constitutes the stimulus to the formation of plasma cells. However, it still remains unknown whether the macrophages that phagocytize the antigen transform into plasmablasts themselves, or whether they stimulate other cells from their surroundings (lymphocytes or reticulum cells) to such a transformation. In tissue culture experiments Roberts, 1955) macrophages, having phagocytized antigens, failed to transform into plasma cells and did not produce antibodies.

As to the histology of the plasma-cell reaction several authors have pointed to the perivascular localization of the plasma cells (Fagraeus, 1948; Frank, 1913; Huebschmann, 1913; Marshall and White, 1950) not only in the spleen but also in connective tissues elsewhere in the body. In our experiments the plasma cell aggregates in the spleen were found to have a very characteristic localization—alongside the border of white pulp lymphoid tissue and surrounding the smallest arterioles emerging from the white pulp strands. Following the arterial pattern the compact lymphoid sheath changes into a plasma-cell sheath upon the branching into the terminal arterioles.

This anatomical situation probably accounts for the small amount of antibody production found in our explants of "white pulp" of the spleen. In preparing white pulp material separately, a slight contamination with plasma cell aggregates is clearly unavoidable as a consequence of the topographical relationships mentioned.

It would seem highly improbable that the white pulp itself produces antibody. The reaction of the white pulp, observed after immunization, i.e., the development of typical "reaction" centers in the Malpighian corpuscles, does not coincide with the formation of immune substances (Ehrich and Harris, 1942). The beginning and the highest development of these reaction centers is found after the beginning and highest rate of antibody formation. It is not very likely therefore (See also Marshall and White, 1950; Ringertz and Adamson, 1950) that these centers form antibodies, as has been previously suggested by Hellman. Moreover the observation mentioned in a previous publication (Thorbecke and Keuning, 1953), that antibody formation in vitro occurs in the bone marrow, while lymph follicles with "reaction" centers were not found in this organ, proves that lymph follicles are not essential for the formation of immune substances. On the other hand, it seems almost sure that these centers have some function in the immunization process in the broad sense of the word, the exact nature of which still remains to be elucidated.

The liver.—In view of the finding of antibody formation, correlated to the proliferation of plasma cells, in the bone

marrow and the spleen it seems justified to assume that in the case where antibody formation in the liver was found, this is to be attributed to the observed proliferation of plasma cells in the periportal connective tissue. Similar changes in the interstitium of the liver were observed by Braude and Spink (1951) in brucella infections of splenectomized rats and by Löwenhaupt (1945) in patients suffering from myelomatosis with general plasmocytosis. At this point we should like to draw attention to the possibility that the observation that antigens are found in the mitochondrial fraction of liver tissue, (Crampton and Haurowitz, 1950) is more likely to be related to the detoxifying action than to any antibody-forming activity of the liver parenchyme.

γ globulin formation.—As has already been described, γ globulin production in vitro could only be demonstrated under conditions in which antibody formation was actively stimulated and demonstrable in the same cultures. Together with the cytologic appearance of immature plasma cells, suggestive of active protein synthesis, and the increase of γ globulins in the blood plasma during antibody formation, this result leaves little doubt that the formation of antibodies is an active synthesis of antibody protein in reaction to antigen. The possibility of a conversion of pre-existent normal γ globulin into immune globulin can be ruled out. This is in keeping with the results of Gros et al (1952), who, with the aid of radioactive labels, showed that injected homologous γ globulins during an active immunization were not directly converted into antibodies (see also Green and Anker, 1954). It is evident, however, that also in non-immunized normal animals a constant γ globulin production must be present in view of the more or less definite concentration of this protein in the blood plasma (Myant, 1952). Apparently our technique was not sensitive enough to demonstrate this normally occurring γ globulin production in the cultured organs.

In contrast with administration of antigens, the loss of appreciable amounts of plasma proteins did not stimulate the formation of γ globulins, at least not to such a degree as to be demonstrable with our method; nor did it induce the formation of plasma cells.

These data together suggest the possibility that all γ globulins might, in fact, be immune globulins, or in other words *that "normal" γ globulins might not exist at all.** In that case both plasma cells and γ globulins would only arise after the penetration of antigens into the body. The available data in the literature do not seem to contradict such a hypothesis.

Most diseases in which a high content of γ globulin is found in the serum are infectious or allergic diseases. Here the increase of the γ globulins is readily explained as resulting from antibody formation. In liver cirrhosis, however, the appreciable increase in γ globulin in the serum cannot be easily accounted for in this way. There is not much evidence of an infectious cause of this disease, and the high γ globulin content is often said to be a compensation of the low albumin content of the serum (Whitman et al, 1950). It has also been shown that this high γ globulin content results from enhanced formation of γ globulin, as there is a normal rate of breakdown of this protein in liver cirrhosis patients (Eisenmenger and Slater, 1953; Havens et al, 1953). Havens et al (1951) found, however, that after an immunization

* Exactly the same may hold true not only for the γ globulins, but also for a part of the β globulins (Oncley, 1953), known to be associated with antibodies (Enders, 1944; see also Miller, 1954b.)

with diphtheria toxoid, patients with chronic hepatic diseases formed, on the average, three times as much antibody as controls. The cause for this finding was not clear, but it may very well be related to or actually be the cause of the high serum γ globulin levels in these patients. It is also known that in liver cirrhosis there is, like in infectious diseases, a distinct proliferation of plasma cells in spleen and bone marrow, sometimes also in the liver (Bing, 1940). On the other hand, in the disease "agammaglobulinemia" of humans, it has been found that plasma cells were virtually absent from the organs (Good, 1955).

Experiments of Richter (1952) suggest that during horse serum immunization γ globulin formation but not antibody formation is enhanced by the injection of yeast ribonucleic acid. In his work, however, the formation of nonprecipitating antibodies as a cause for the high γ globulin levels has not been excluded.

It might be argued that the constant γ globulin production sustaining normal γ globulin levels in normal, nonimmunized individuals cannot just represent antibody formation. However, the organs of "normal" animals are not sterile (Schweinburg and Sylvester, 1953) and, for example, the intestinal flora constantly induces the production of immune substances. Also long lasting immunities, such as resulting from yellow fever and measles, demand a constant production of antibodies.

Lastly, the relationships between antibody formation and plasma cells on the one hand and serum γ globulin levels on the other in the newborn are very suggestive of the hypothesis that "normal" γ globulin does not exist. In some experiments with young rabbits not reported in detail here, we investigated the time of appearance of plasma cells in relation to the serum γ globulin level. In rabbits, like in the human, maternal γ globulin passes through the placenta, resulting in approximately equal concentrations of γ globulins (and antibodies) in the blood of mother and newborn. After birth the γ globulin level in the newborn gradually falls at first; subsequently it increases again, apparently through γ globulins produced by the young animal itself (Moore et al, 1949). In our experiments the lowest concentrations were found in the 2 and 4 week age groups. Now, in accordance with Maximow (1907) and Schridde (1911), we failed to find any plasma cells in the tissues immediately after birth. The first appearance of these cells was at the age of 1 to 2 weeks, and they were first seen in the intestinal wall and the spleen.

Undoubtedly it will prove difficult to distinguish experimentally between the formation of a "normal" γ globulin and of immune γ globulins. It does not seem quite feasible at the moment to inhibit antibody formation selectively in normal animals, or to raise animals that are completely free from antigenic contacts.

SUMMARY

Histological observations on organs showing antibody formation in vitro are described. The localization of plasma cells was found to be mainly perivascular, particularly in the spleen, and/or in the vicinity of lymphoid tissue.

The occurrence of antibody production in vitro in liver tissue could be correlated to a proliferation of plasma cells in the espaces portes. Both appeared after intravenous paratyphoid B immunization in splenectomized rabbits and after horse serum immunization in normal rabbits.

After immunization, a close parallelism could be demonstrated between the formation of antibodies and of γ globu-

lin in vitro in various organs, particularly in bone marrow and splenic red pulp. With the techniques used, no significant γ globulin formation could be found in organs of nonimmunized rabbits. Nor could γ globulin production be demonstrated in the organs of animals that had been subjected to plasmapheresis. No proliferation of plasma cells was found after plasmapheresis. On electrophoretic analysis of the serum proteins, it seemed that after plasmapheresis the regeneration of γ globulin was slower than of the other serum proteins.

The absence of the formation of both plasma cells and γ globulin before birth is discussed and some additional experiments on this subject are briefly mentioned.

The hypothesis is brought forward that all γ globulins in the blood plasma are antibodies and a discussion on the possibility of the non-existence of "normal" γ globulin is presented.

REFERENCES

Bing, J. 1940, Acta med. Scandinav. **103**: 565–583.

Brachet, J. 1953, Quart. J. Micr. Sc. **94**: 1–10.

Braude, A. I. and Spink, W. W. 1951, J. Infect. Dis. **89**: 272–276.

Crampton, C. F. and Haurowitz, F. 1950, Science, **112**: 300–302.

Ehrich, W. E. and Harris, T. N. 1942, J. Exper. Med. **76**: 335–348.

Eisenmenger, W. J. and Slater, R. J. 1953, J. Clin. Investigation, **32**: 564–565.

Enders, J. F. 1944, J. Clin. Investigation, **23**: 510–530.

Fagraeus, A. 1948, Acta med. Scandinav. suppl. 204.

Frank, A. 1913, Verhandl. d. deutsch. pathol. Gesellsch. **16**: 115–126.

Good, R. A. 1955, J. Lab. & Clin. Med. **46**: 167–181.

Green, H. and Anker, H. S. 1954, Biochim. et Biophys. Acta, **13**: 365–373.

Gros, P., Coursaget, J. and Macheboeuf, M. 1952, Bull. Soc. Chim. Biol. **34**: 1070–1077.

Haurowitz, F. 1953, in: The Nature and Significance of the Antibody Response, Columbia Univ. Press, p. 3.

Havens, W. P., Dickeinsheets, J., Bierly, J. N. and Eberhard, T. P. 1953, J. Clin. Investigation, **32**: 573.

Havens, W. P., Shaffer, J. M. and Hopke, C. J. 1951, J. Immunol. **67**: 347–356.

Hayhoe, F. G. J. and Smith, D. R. 1951, J. Clin. Path. **4**: 47–54.

Huebschmann. 1913, Verhandl. d. deutsch. pathol. Gesellsch. **16**: 110–115.

Keuning, F. J. 1954, Chem. Weekbl. **50**: 702–704.

Keuning, F. J. and van der Slikke, L. B. 1950, J. Lab. & Clin. Med. **36**: 167–182.

Klein, H. and Block, M. 1953, J. Hematol. **8**: 1034–1041.

Koch, K. 1913, Virch. Arch. **211**: 321–331.

Loiseleur, J. 1951, Compt. rend. Acad. d. sc. **233**: 504–506.

Lowenhaupt, E. 1945, Am. J. Path. **21**: 171–185.

Macheboeuf, M., Rebeyrotte, P., Dubert, J. M. and Brunerie, M. 1953, Bull. Soc. Chim. Biol. **35**: 334–345.

Majoor, C. L. H. 1947, J. Biol. Chem. **169**: 583–594.

Marshall, A. H. E. and White, R. G. 1950, Brit. J. Exper. Path. **31**: 157–174.

Maximow, A. 1907, Folia haemat. **4**: 611.

Miller, L. L. and Bale, W. F. 1954a, J. Exper. Med. **99**: 125–132.

Miller, L. L., Bly, C. G. and Bale, W. F. 1954b, J. Exper. Med. **99**: 133–153.

Miller, L. L., Bly, C. G., Watson, M. C. and Bale, W. F. 1951, J. Exper. Med. **94**: 431–453.

Moeschlin, S. 1951, Spleenpuncture, London.

Moore, D. H., DuPan, R. M. and Buxton, C. L. 1949, Am. J. Obstet. & Gyn. **57**: 312–322.

Mountain, I. M. 1955, J. Immunol. **74**: 270–277.

Mushett, C. W., Stern, K. G. and Silber, R. H. 1949, Am. J. M. Sc. **218**: 58–64.

Müting, D. 1951, Arch. f. klin. Med. **198**: 686–697.

Myant, N. B. 1952, Clin. Sc. **11**: 191–201.

Oncley, J. L. 1953, in: Blood Cells and Plasma Proteins, New York, p. 180.

Proom, H. 1943, J. Path. Bact. **55**: 419–426.

Ranney, H. M. and London, I. M. 1951, Fed. Proc. **10**: 562–563.

Richter, G. W. 1952, J. Exper. Med. **96**: 331–346.

Ringertz, N. and Adamson, C. A. 1950, Acta Path. Microbiol. Scandinav. suppl. **86**.

Roberts, K. B. 1955, Brit. J. Exper. Path. **36**: 199–204.

Scheer, J. van der, Bohnel, E., Clarke, F. H. and Wyckoff, R. W. G. 1942, J. Immunol. **44**: 165–174.

Scheer, J. van der, Lagsdin, J. B. and Wyckoff, R. W. G. 1941a, J. Immunol. **41**: 209–223.
Scheer, J. van der, Wyckoff, R. W. G. and Clarke, F. H. 1941b, J. Immunol. **40**: 173–177.
Schweinburg, F. B. and Sylvester, E. M. 1953, Proc. Soc. Exper. Biol. & Med. **82**: 527–530.
Schlienger, C. 1945, Schweiz. Ztschr. f. Path. u. Bact. **8**: 222–237.
Schridde, H. 1911, in: Aschoff's Lehrbuch der Path. Anat., Jena, p. 124.
Thorbecke, G. J. and Keuning, F. J. 1953, J. Immunol. **70**: 129–134.
Vink, H. H. 1947, Tijdschr. v. Diergeneesk. **72**: 212–215.
Weichselbaum, T. E., 1946, Am. J. Clin. Path. **16**: 40–49.
Whitman, J. F., Rossmiller, H. R. and Lewis, L. A. 1950, J. Lab. & Clin. Med. **35**: 167–180.
Wuhrmann, F. 1947, in: Die Bluteiweiszkörper des Menschen, Basel, p. 310.

THE FLOCCULATION TEST WITH A PURIFIED ANTIGEN IN THE DIAGNOSIS OF TRICHINOSIS IN HUMANS

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A rapid slide flocculation test for the diagnosis of trichinosis was described by Bozicevich and his coworkers. [1,2] This test, which employed as antigen a saline extract of *Trichinella spiralis* larvae adsorbed onto bentonite particles, was reported to be equally as sensitive and specific as the complement-fixation reaction. However, a complete evaluation of the test in relation to actual infection with this nematode in humans was not done.

A serologically active fraction of *Trichinella spiralis* larvae was isolated by Melcher.[3] This acid soluble protein fraction, when used in the flocculation test on rabbits, hogs and humans, was found to be at least as sensitive and specific as the whole larval antigen.[4] In addition, this antigen can be stored for long periods of time without deterioration, and it permits the formation of more distinct flocculating particles which make the test easier to read. Therefore, an attempt was made to compare the flocculation test, using this purified antigen, with the complement-fixation test as routinely employed in the diagnosis of trichinosis in humans. The latter test was performed by the staff of the Serology Laboratory of the Communicable Disease Center, under the direction of Dr. J. Schubert, who has kindly allowed us access to the results.

MATERIALS AND METHODS

The purified protein fraction antigen for the flocculation reaction was prepared with strict adherence to the method described by Melcher.[3] The flocculation test was performed according to the published method.[1] The serum samples were, for the most part, serially diluted, 1:5, 1:10, 1:20, etc., until a negative reaction was obtained. The whole larval antigen for the complement-fixation test was prepared as described by Bozicevich and his coworkers, with the exception only that 5 ml of settled larvae were used per 100 ml of antigen instead of 2 ml.[4]

A total of 1331 human serums were tested both by the complement-fixation and the flocculation reaction. All the serums were coded, on arrival, and tested immediately by the complement-fixation reaction. A different person tested them, as a rule, one day later by the flocculation reaction. To obviate bias, the name of the patient, source of the specimen, and the result of the complement-fixation test were unknown to the worker at the time the flocculation test was performed. Merthiolate in the amounts normally employed as serum preservatives had been added to some of the serums before they were sent to the Communicable Disease Center. Other serums showed evidence of bacterial contamination on arrival. Although most of the heavily contaminated serums were anticomplementary when

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1. Bozicevich, J., Tobie, J. E., Thomas, E. H., Hoyem, H. M. and Ward, S. B. 1951, A rapid flocculation test for the diagnosis of trichinosis. Pub. Health Rep. **66**: 806–814.
2. Thomas, E. H., Bozicevich, J. and Hoyem, H. M. 1953, Flocculation reactions in rabbits experimentally infected with *Trichinella spiralis*. J. Infect. Dis. **92**: 89–96.
3. Melcher, L. R. 1943, An antigenic analysis of *Trichinella spiralis*. J. Infect. Dis. **73**: 31–39.
4. Sadun, E. H. and Norman, L. 1955, The use of an acid soluble protein fraction in the flocculation test for the diagnosis of trichinosis. J. Parasitol. In press.

tested by the complement-fixation reaction, there was no interference with the readings of the flocculation reaction.

In an effort to evaluate the serological results, letters and questionnaires were sent to the physicians whose patients' serums had been examined, asking for certain information related to the history of the patient, other laboratory findings, clinical symptoms and the tentative diagnosis. Muscle biopsy, eosinophilia, facial edema and the physician's clinical diagnosis were taken as means of comparison. Although the proportion of eosinophiles is normally 3 or 4% of the total white cell count, the 10% level was taken as the dividing line between patients with or without frank eosinophilia. This higher figure was chosen to eliminate several borderline cases. In all, 206 questionnaires, completely filled out, were returned to us.

There were more serums examined than patients because, occasionally, two or more serums were drawn from the same patient at different times. In relating the results of serological reactions to clinical and other laboratory findings, a difficulty was encountered in realizing that some serums which had been obtained from the same patients at different times gave different reactions. In those few instances in which a choice of the results had to be made, an arbitrary time was taken as the dividing line. The specimen drawn on the day closest to 30 days following the onset of symptoms was selected as representative of that patient's condition.

RESULTS

The 1331 serums tested by the flocculation and complement-fixation reactions were obtained from about 1200 patients in 41 states, the District of Columbia, Canada and the Canal Zone. Clinical and other laboratory information was obtained from 206 patients in 26 states. The age and sex distributions of these patients are shown in table 1. Of 206 individuals, 135, or 65.6%, were males, and 104, or 50.5%, were under 10 years of age.

TABLE 1.—*Age and sex distribution of patients from physicians' questionnaires.*

Age in years	Sex			Total
	Male	Female	Not stated	
0– 9	65	36	3	104
10–19	6	2		8
20–29	26	9		35
30–39	18	8		26
40–49	10	4		14
50–59	6	8		14
60 plus	4	1		5
Total	135	68	3	206

TABLE 2.—*Results of flocculation and complement-fixation reactions of all the serums.*

Results	Flocculation test		Complement fixation test	
	Number	Percent	Number	Percent
Positive	315	24	219	16
Negative	1016	76	985	74
Anticomplimentary			127	10
Total	1331	100	1331	100

The data obtained from all the serums are summarized in table 2. Of 1331 specimens, 315, or 24%, were positive by the flocculation test and 219, or 16%, were positive by the complement-fixation test. The others were either negative or anticomplementary. The results obtained by the flocculation and complement-fixation reactions are compared in table 3. A total of 1051 serums, or 79%, gave similar results with both tests, and 153 serums, or 11%, gave contrasting results. Of the 127 serums which were anticomplementary with the complement-fixation reaction, 31 were positive by the flocculation test, whereas 96 were negative.

In the attempt to evaluate the results of the flocculation and complement-fixation reactions in relation to actual infection with *T. spiralis*, data from the questionnaires were analyzed. The most common symptoms of trichinosis are myositis, diarrhea, fever, facial edema

TABLE 3.—*Comparison of results by the flocculation and complement-fixation reactions.*

Flocculation	Complement-fixation	Number	Percent
Positive	Positive	175	13
	Negative	109	8
	Anticomplementary	31	2
Negative	Positive	44	3
	Negative	876	66
	Anticomplementary	96	7
Total		1331	99*

* Since these percentages were in whole numbers, their nearest approximations add up to 99 instead of 100.

TABLE 4.—*Comparison of flocculation and complement-fixation reactions in relation to clinical and other laboratory findings.*

Serological reactions of patients	Facial edema			Eosinophilia			Biopsy			Physicians' tentative diagnosis		
	Total reported	No. with symptoms	Percent with symptoms	Total reported	No. with 10% or above	Percent W/10% or above	Total reported	Number positive	Percent positive	Total reported	Number with trichinosis	Percent with trichinosis
Total patients	206	102	50	160	133	83	52	25	48	206	115	56
Tot. floc. pos.	142	81	57	115	106	92	42	23	52	142	103	73
Tot. floc. neg.	64	21	33	45	27	60	10	2	20	64	12	19
Tot. CF pos.	102	61	60	84	75	89	27	13	48	102	78	76
Tot. CF neg.	86	34	40	59	45	76	20	9	45	86	29	34
Tot. CF AC	18	7	39	17	13	76	5	3	60	18	8	44
Floc. pos. CF pos.	91	58	64	74	70	95	22	11	50	91	73	80
Floc. neg. CF pos.	11	3	27	10	5	50	5	2	40	11	5	45
Floc. pos. CF neg.	40	16	40	31	27	87	16	9	56	40	22	55
Floc. neg. CF neg.	46	18	39	28	18	64	4	0	0	46	7	15
Floc. pos. CF AC	11	7	64	10	9	90	4	3	75	11	8	73
Floc. neg. CF AC	7	0	0	7	4	57	1	0	0	7	0	0

and eosinophilia.[5–10] Since myositis, diarrhea, and fever could pass unnoticed and/or could be caused by several common conditions other than trichinosis, no attempt was made to relate these symptoms to the serological results. On the other hand, an attempt was made to interpret the serological findings in relation to the physician's tentative diagnosis reached on the basis of a history of the ingestion of contaminated food, physical examination, and laboratory tests other than serological tests. The results of muscle biopsy were available on 52 patients. Table 4 shows that of the patients with positive flocculation reactions, 57% had facial edema, 92% had eosinophilia, and 73% had been diagnosed by their physicians as having trichinosis. Trichinella larvae were observed in the muscle specimens of 52% of the people examined in this group. Conversely, of the patients whose serums gave negative flocculation reaction, 33% had facial edema, 60% had eosinophilia, and 19% had been tentatively diagnosed by their physicians as having trichinosis. Only 2 of the biopsies among patients in this group were positive. Essentially similar results were obtained with the complement-fixation reaction. The serums that gave positive reactions came mainly from people who had been tentatively diagnosed as having trichinosis. On the other hand, only 34% of the patients

5. Vener, H. I. and Stevens, G. M. 1938, Trichiniasis. Report of an outbreak of 25 cases. Los Angeles City Board of Health Comm. Bull. **33:**
6. Wyrens, R. G., Tillish, J. H. and Magath, T. B. 1941, Trichinosis; report of 19 cases of clinical infection and 21 cases of asymptomatic infection. J.A.M.A. **117:** 428–432.
7. Kirk, R. C. 1942, The causes of eosinophilia. New International Clinics. **1:** 219–233.
8. Parrisius, W., Lampe, G., Romer, W. and Honighaus, L. 1942, Erfahrungen während einer Trichinose-Epidemia. Deut. Militärarzt. **7:** 198–209.
9. Weitz, W. 1931, Zur Symptomatologie der Trichinose. Ztschr. f. Klin. Med. **116:** 144–173.
10. Oppenheim, J. M., Whims, C. B. and Frisch, A. W. 1946, Clinical and laboratory observations on 256 cases of trichinosis. Bull. U. S. Army Med. Dept. **6:** 581–593.

whose serums were negative by the complement-fixation test had been tentatively diagnosed, on clinical grounds, as having trichinosis. Larvae were recovered from 9, or 45%, of those in this group on which a muscle biopsy was performed.

In comparing the results of the flocculation with those of the complement-fixation test (table 4) it was observed that those serums giving parallel results came from patients who, for the greatest part, had high eosinophilia, facial edema, and a tentative diagnosis of trichinosis. Larvae were observed from 50% of those patients in this group who were submitted to a biopsy. The serums which gave negative reactions with both tests came, for the most part, from patients whose physicians' tentative diagnoses were other than trichinosis. Larvae were not observed in the muscles of any person in this group and facial edema was reported in 39%. The serums which gave contrasting results by the two tests came from individuals whose clinical characteristics and laboratory findings were somewhat intermediate between the groups with positive and negative reactions by both tests.

DISCUSSION

Results of this study in which 1331 serums were tested by the flocculation and complement-fixation reactions indicate that there was close agreement between the two tests in 79% and contrasting results in 11% of the serums examined. The remaining 10% were anticomplementary.

In order to obtain direct evidence as to the validity of these serological tests, the results obtained on 206 patients were related to clinical and other laboratory findings. Our series includes abnormally high percentages of people with eosinophilia and facial edema even among those whose serological results, history, and clinical findings tend to rule out the possibility of having contracted trichinosis. This is due to the fact that the specimens analyzed in this paper do not represent a random sample but, on the contrary, were sent to the Communicable Disease Center Laboratory because the physician had observed symptoms such as eosinophilia and facial edema which are compatible with, although not necessarily pathognomonic of, trichinosis. Under these circumstances, the high percentage of eosinophilia, facial edema, or tentative diagnosis of trichinosis cannot be used as definite proof of the accuracy of the resulting serological diagnosis. Actually, the only critical data having significant bearing on the results of the serological diagnosis are the biopsy results. These are significant for two reasons: (1) They are completely objective data and (2) in all probability, most of them were not available until after the serums had been taken and did not, in themselves, lead to the taking of them.

There are roughly the same percentages of patients with facial edema, eosinophilia, and clinical diagnosis of trichinosis among the flocculation positives as there are among the complement-fixation positives. In view of the above considerations, this could be taken only as a suggestion of the correlation between serological positivity and trichinosis. This suggestion is strengthened by the fact that roughly the same proportion of patients had a positive biopsy among those with a positive complement-fixation as had those with a positive flocculation reaction. The only consistent difference between the two tests is that a higher percentage of people with a positive biopsy (as well as facial edema, eosinophilia, and physcians' diagnoses) was encountered in the group giving a negative complement-fixation reaction than in

that giving a negative flocculation reaction.

Some of the serums which gave contrasting results with the two tests came from individuals who had a history of having been exposed to infective meat only a short time before examination. Repeat specimens, at a later date, from these individuals were positive with both tests. Other serums which gave contrasting results came from patients who had presumably been exposed to infection several months before. Relatively low titers were obtained from all of these serums. Therefore, the difference in the two reactions may be primarily quantitative. When the concentration of antibodies was so low as to be barely detectable by one test, it was occasionally missed by the other. In the light of the above considerations, it seems possible that most of the serums which gave contrasting results were from people who had contracted the infection with this parasite, but whose antibody level was low either because of the fact that the infection was very mild in character or because serum had been collected before or long after the peak of antibody titer was reached.

Although only one positive biopsy was reported in the group of serums which gave negative reactions, 15 of the people whose serums gave positive reactions with both tests had a negative biopsy. This is compatible with the view expressed by previous authors that in mild infections a negative biopsy is not uncommon.

In general, the results indicate a close correlation between the flocculation and the complement-fixation reactions. The flocculation test can be performed in a short time by personnel who are not necessarily highly trained, it is much easier to read, it permits examinations of serums which are anticomplementary to the complement-fixation reaction, and it utilizes an antigen which can be stored over long periods of time. Because of these considerations, we regard it as the test of choice.

SUMMARY

Bentonite flocculation tests with an acid soluble protein fraction antigen and complement-fixation tests with a crude larval antigen gave comparable results in 1331 human serums. The results of the two tests in 206 patients were compared with observations made on clinical grounds, and with other laboratory findings. A mutual relationship between serological findings and clinical trichinosis was observed.

On the basis of the results of this work and in view of ease of performance, it is believed that the flocculation test can be used instead of the complement-fixation test for the serological diagnosis of trichinosis in human beings.

THE ACTIVITY OF TETRACYCLINE ON FELINE PNEUMONITIS VIRUS INFECTION OF CHICK EMBRYOS

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It has been demonstrated that chlortetracycline and oxytetracycline are effective against viruses of the psittacosis-lymphogranuloma group in experimental infections of mice[1,2] and chick embryos[3–6] and that the tetracyclines have been successfully employed clinically.[7–10] Chlortetracycline has become the drug of choice in the treatment of human infections caused by these agents. Recently these drugs have shown some promise in field trials in the chemotherapy of natural psittacosis and ornithosis.[11,12]

In experimental infections of laboratory animals and chick embryos, chlortetracycline prolongs the period of survival of the infected animals, thus suppressing but not completely inhibiting the multiplication of these viruses in vivo.[1,3,6] Since it has been demonstrated that chlortetracycline is unstable in vitro at 37 C at neutral or slightly alkaline pH, or in the presence of protein,[13,14]

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1. Kneeland, Y., Jr. and Price, K. M. 1950, Treatment with chloramphenicol, aureomycin and terramycin of the pneumonia caused by feline pneumonitis virus. J. Immunol. **65**: 653–660.
2. Loosli, C. G., Hamre, D., Ritter, G. and Berlin, B. S. 1954–1955, Experimental airborne mouse pneumonitis virus (MPV) infections in mice. II. The prophylactic and therapeutic effect of sulfadiazine and antibiotics. Antibiotics Ann. pp. 474–489.
3. Allen, E. G., Girardi, A. J., Sigel, M. M. and Klein, M. 1953, Studies on the psittacosis-lymphogranuloma group. III. Effect of aureomycin on propagation of virus in the chick embryo. J. Exper. Med. **97**: 783–795.
4. Gogolak, F. M. and Weiss, E. 1950, The effect of antibiotics on agents of the psittacosis-lymphogranuloma group. II. Effect of aureomycin. J. Infect. Dis. **87**: 264–274.
5. Hurst, E. W., Peters, J. M. and Melvin, P. 1950, Therapy of experimental psittacosis and lympho-granuloma venereum (inguinale). 1. Comparative efficacy of penicillin, chloramphenicol, aureomycin and terramycin. Brit. J. Pharm. & Chemotherap. **5**: 611–624.
6. Wong, S. C. and Cox, H. R. 1948, Action of aureomycin against experimental rickettsial and viral infections. Ann. N. Y. Acad. Sc. **51**: 211–217.
7. Brainerd, H., Lennette, E. H., Meiklejohn, G., Bruyn, H. B., Jr. and Clark, W. H. 1949, The clinical evaluation of aureomycin. J. Clin. Investigation **28**: 992–1005.
8. Fitz, R. H., Meiklejohn, G. and Baum, M. D. 1955, Psittacosis in Colorado. Am. J. M. Sc. **229**: 252–261.
9. Green, T. W. 1950, Aureomycin therapy of human psittacosis. J.A.M.A. **144**: 237–238.
10. Wright, L. T., Sanders, M., Logan, M. A., Prigot, A. and Hill, L. M. 1948, Aureomycin, a new antibiotic with virucidal properties. 1. A preliminary report on successful treatment in twenty five cases of lymphogranuloma venereum. J. A. M. A. **138**: 408–412.
11. Meyer, K. F. and Eddie, B. 1955, Chemotherapy of natural psittacosis and ornithosis. Field trial of tetracycline, chlortetracycline and oxytetracycline. Antibiot. & Chemotherap. **5**: 289–299.
12. Meyer, K. F. and Eddie, B. 1954–1955, Chemotherapy of natural psittacosis and ornithosis. Field trial of tetracycline (polyotic HCl), chlortetracycline and oxytetracycline. Antibiotics Ann. pp. 544–555.
13. Price, C. W., Randall, W. A. and Welch, H.

the failure to completely inhibit multiplication of the virus in vivo might be related to this instability. When present in sufficient concentration chlortetracycline inhibits the virus completely; but due to a gradual destruction of the antibiotic, its concentration decreases to a point where the virus starts to multiply slowly. Subsequently, the level of chlortetracycline is so greatly reduced that virus multiplication proceeds to an even greater extent. Of the tetracycline antibiotics, tetracycline is the most stable of the group.[15,16] Therefore, this drug might be expected to exhibit even greater activity against members of the psittacosis-lymphogranuloma group of viruses. Also, an antibiotic with greater stability than chlortetracycline or penicillin, for example, would be useful in the study of such problems as the development of drug resistance or the effect of the drug on various stages of the developmental cycle of these viruses.

Since there have been few reports[8,11,12] concerning the effect of tetracycline on members of this group of viruses, an investigation was undertaken to study the activity of tetracycline on feline pneumonitis in infections of chick embryos.

MATERIALS AND METHODS

Virus.—The virus used throughout these studies was an egg-adapted strain of feline pneumonitis virus.[17] A pool of virus was prepared in the following manner: 6-day-old embryonated eggs were infected with feline pneumonitis via the yolk sac route, and the yolk sacs and yolk fluid of moribund embryos were harvested when one-half the embryos had died. Each yolk sac was emulsified separately by grinding with sand in a mortar and pestle under sterile conditions. Using yolk fluid obtained from the same embryo, a 20% suspension of this material was prepared. After determining that these preparations were free from bacterial contamination by gram stain and sterility test in thioglycollate broth, the yolk sac suspensions were pooled and lightly centrifuged at 1500 r.p.m. for 5 minutes. The supernatant was collected and diluted with inactivated horse serum and Bacto tryptose broth to give a 10% suspension of virus. This suspension was distributed in 1.5 ml quantities in 4 ml screwcap vials and stored in a dry ice box at −76 C. This suspension killed chick embryos via the yolk sac route when 0.5 ml of a dilution of 10^{-7} was used. The method of Reed and Muench [18] was employed throughout for determination of the LD_{50} titer. The morphology and developmental cycle of feline pneumonitis virus is discussed by Gordon.[19]

Antibiotics.—Aureomycin hydrochloride (Lederle Laboratories)* buffered with sodium glycinate was obtained in 100 mg sterile vials as distributed for hospital use for intravenous injection. Terramycin hydrochloride (Chas. Pfizer and Co.) buffered with sodium glycinate, and tetracycline hydrochloride (J. B. Roerig and Co.) buffered with ascorbic acid were obtained in 250 mg vials for intravenous injection. In preparation for use, the drugs were dissolved in sterile distilled water, and subsequent dilutions were made in sterile Bacto tryptose broth to obtain the desired amount of antibiotic in 0.5 ml of fluid. Infected controls received 0.5 ml of sterile tryp-

1948, Bacteriological studies of aureomycin. Ann. N. Y. Acad. Sc. **51**: 211–217.

14. Paine, T. F., Jr., Collins, H. S. and Finland, M. 1949, Bacteriologic studies on aureomycin. J. Bact. **56**: 489–497.
15. English, A. R., P'an, S. Y., McBride, T. J., Gardocki, J. F., Van Halsema, G. and Wright, W. A. 1953–1954, Tetracycline-microbiologic, pharmacologic and clinical evaluation. Antibiotics Ann. pp. 70–80.
16. Wright, S. S., Purcell, E. M., Love, B. D., Jr., Mou, T. W., Kass, E. H. and Finland, M. 1953–1954, Clinical and laboratory observations on tetracycline. Antibiotics Ann. pp. 92–97.

* The author wishes to thank Dr. Stanton M. Hardy, Director, Human Clinical Research, Lederle, Division of American Cyanamid Company, Pearl River, N. Y. for the generous supply of chlortetracycline hydrochloride.

17. Baker, J. A. 1944, A virus causing pneumonia in cats and producing elementary bodies. J. Exper. Med. **79**: 159–171.
18. Reed, L. J. and Muench, H. 1938, A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**: 493–497.
19. Gordon, F. B. 1955, The psittacosis virus: A general survey. Psittacosis Diagnosis, Epidemiology and Control. Rutgers Univ. Press, pp. 9–21.

TABLE 1.—*The effect of chlortetracycline, oxytetracycline, and tetracycline on feline pneumonitis virus infection of 6-day-old chick embryos.*

Antibiotic	Amount of antibiotic, mg	Average day of death of embryos	Delay in death (days) of treated vs. control embryos	Alive / total	Percentage survival
Control	0.0	4.9	0	0/24	0
Chlortetracycline	1.0			20/21	95
	0.5			22/23	96
	0.25			20/21	95
	0.125	9.9	5.0	2/23	9
	0.0625	7.0	2.1	0/21	0
	0.031	5.4	0.5	0/22	0
Oxytetracycline	1.0			22/22	100
	0.5			20/21	95
	0.25			21/21	100
	0.125			21/21	100
	0.0625			20/21	95
	0.031			18/19	95
Tetracycline	1.0			23/23	100
	0.5			19/21	91
	0.25			19/20	95
	0.125			15/15	100
	0.0625			21/21	100
	0.031			20/21	95

tose broth in place of a similar volume of antibiotic.

Chick embryos.—Six-day-old-white Leghorn chick embryos were used. Groups of 20 to 30 embryos were employed in each experiment. Both the virus and the antibiotic were injected in 0.5 ml of fluid into the yolk sac. For this purpose 5 ml syringes fitted with 1¼ inch, 20 gauge needles were used. After injection the eggs were sealed with collodion, incubated at 36 C, and candled daily for 12 days. The time of death of embryos was determined and the average day of death (ADD) was calculated as described by Golub.[20] Death of embryos within the first 2 days after injection was considered nonspecific and these were not included in the final calculations. The number of living to the total number of embryos employed and the percentage of embryos alive in each experimental group at the conclusion of the experiment were also determined.

Staining.—To ascertain whether death of embryos was due to the presence of virus or bacterial contamination, and whether virus was present in surviving embryos, the yolk sac of the embryo in question was harvested and impression smears prepared. These were stained according to Macchiavello's technique and examined for the presence of virus particles and the absence of bacterial contamination.

20. Golub, O. J. 1948, A single dilution method for the estimation of LD_{50} titers of the psittacosis lymphogranuloma venereum group of viruses in chick embryos. J. Immunol. **59**: 71–82.

RESULTS AND DISCUSSION

The effect of various concentrations of chlortetracycline, oxytetracycline, and tetracycline on feline pneumonitis in the chick embryo.—To compare the activity of the tetracycline antibiotics, 6-day-old chick embryos were each injected with 50,000 LD_{50}'s of feline pneumonitis virus, and 1 hour later groups of 25 of the embryos were given amounts of these antibiotics, varying from 0.031 to 1.0 mg. From the data presented in table 1, it can be seen that oxytetracycline and tetracycline were superior to chlortetracycline in protecting the embryos infected with feline pneumonitis virus, indicating that these two antibiotics inhibited virus multiplication to a greater extent. In contrast to chlortetracycline[4] and penicillin,[21] the activity of tetracycline and oxytetracycline over the concentration range used was not directly proportional to the amount of the drug employed. Levels of these drugs as low as 0.031 mg were just as effective as 1 mg in protecting the em-

21. Weiss, E. 1950, Effect of antibiotics in agents of the psittacosis lymphogranuloma groups. I. Effect of penicillin. J. Infect. Dis. **87**: 249–263.

TABLE 2.—*The influence of the size of the virus inoculum on the activity of tetracycline.*

Amount of antibiotic, mg	Virus concentration, LD_{50}	Average day of death	Delay in death (days) of treated vs. control embryos	Alive / total	Percentage survival
1.0	100,000			22/22	100
0.5				21/21	100
0.25				21/22	96
0.125				19/19	100
0.0625				23/23	100
0.031				20/23	87
0.016		7.1	2.8	1/21	4.7
0.000		4.3	0	0/22	0
1.0	10,000			18/20	90
0.5				17/18	94
0.25				19/20	95
0.125				22/22	100
0.0625				21/22	95
0.031				18/19	95
0.016		8.4	3.2	1/19	5
0.000		5.2	0	0/22	0
1.0	1,000			19/21	91
0.5				20/22	91
0.25				21/21	100
0.125				17/18	94
0.0625				20/22	91
0.031				16/19	84
0.016		9.0	3.0	3/22	14
0.000		6.0	0	0/22	0

bryos for 12 days. Eight to nine times as much chlortetracycline was required to produce the same effect over this period of time. At concentrations below 0.25 mg chlortetracycline could only delay the death of the infected treated embryos. Since chlortetracycline is less stable than either tetracycline or oxytetracycline, this may account for its failure to exhibit activity equal to that of the other two drugs.

The influence of the size of the virus inoculum on the activity of tetracycline.—It was of further interest to examine the efficacy of different concentrations of tetracycline when the size of the virus inoculum was varied. Groups of embryos were inoculated with 100,000, 10,000, and 1000 LD_{50}'s of feline pneumonitis virus, respectively. One hour later, groups of 25 of the embryos were given amounts of tetracycline varying from 0.016 to 1.0 mg. The experimental data are presented in table 2. It is evident that the activity of tetracycline, over a wide range of concentration, is independent of the size of the virus inoculum used. These results compare favorably with those obtained by Gogolak and Weiss,[4] who studied the influence of various inoculums of feline pneumonitis virus on the activity of chlortetracycline. A comparison of the data points to an even greater protection of infected embryos by tetracycline.

When a virus inoculum of 100,000 LD_{50}'s was used the untreated controls died in 4.3 days, whereas embryos receiving as little as 0.016 mg survived for an additional 2.8 days. When the inoculum consisted of 10,000 LD_{50}'s, the ADD of the infected controls was 5.2 and of infected embryos receiving 0.016 mg, 8.4,—a delay of 3.2 days. Control embryos injected with 1000 LD_{50}'s died at 6.0 days; infected embryos receiving 0.016 mg were protected for an additional period of 3 days. It may be noted that regardless of the size of the virus inoculum, higher concentrations of the antibiotic gave nearly complete protection of infected embryos. These results further indicate that tetracycline is highly effective against feline pneumonitis virus.

The effect of tetracycline on virus infection after delayed administration of the drug.—To obtain additional information on the activity of tetracycline, a

study was made to determine the effect of tetracycline on virus infection after a delay in administration of the drug. In previous experiments the antibiotic was administered shortly after introduction of the virus into chick embryos, and, under these conditions, the virus does not have sufficient opportunity to develop in the absence of the antibiotic. A delay in administration of the drug would permit active multiplication of the virus to occur, and thus, with time, there would be an increasing number of viral particles present and of host cells infected and damaged by the developing virus. Such a situation would present a greater challenge to the effectiveness of the antibiotic.

This study was divided into two experiments. In the first, the embryos were infected with 100,000 LD_{50}'s of feline pneumonitis virus. A group of 25 of the embryos was then treated with 0.25 mg tetracycline per embryo at 48, 72, 84 and 96 hours after virus infection. Additional groups of 25 embryos were treated at the same time intervals with 0.025 mg tetracycline per embryo. The 9th group served as infected but untreated controls. The results are presented in figures 1 and 2. It can be seen that 0.25 mg tetracycline possessed

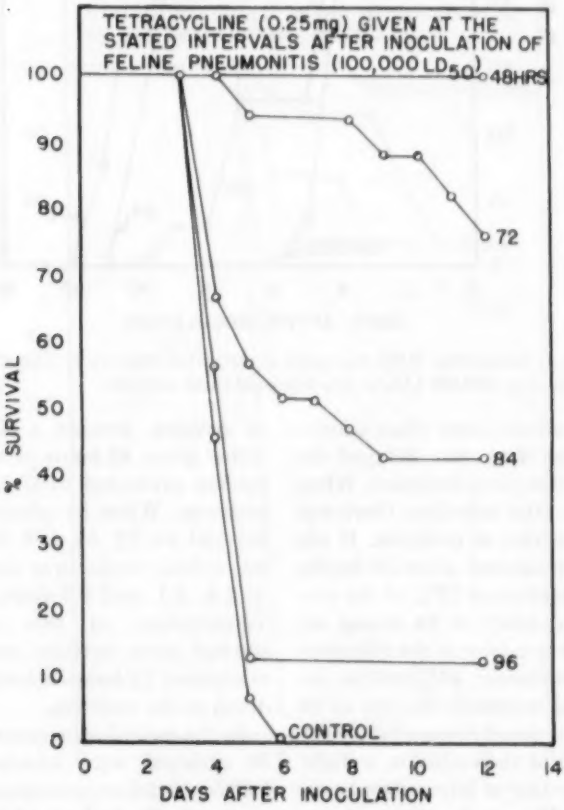

FIG. 1.—Effect of tetracycline (0.25 mg) given at the stated intervals of time after inoculation of feline pneumonitis virus (100,000 LD_{50}'s) into 6-day-old chick embryos.

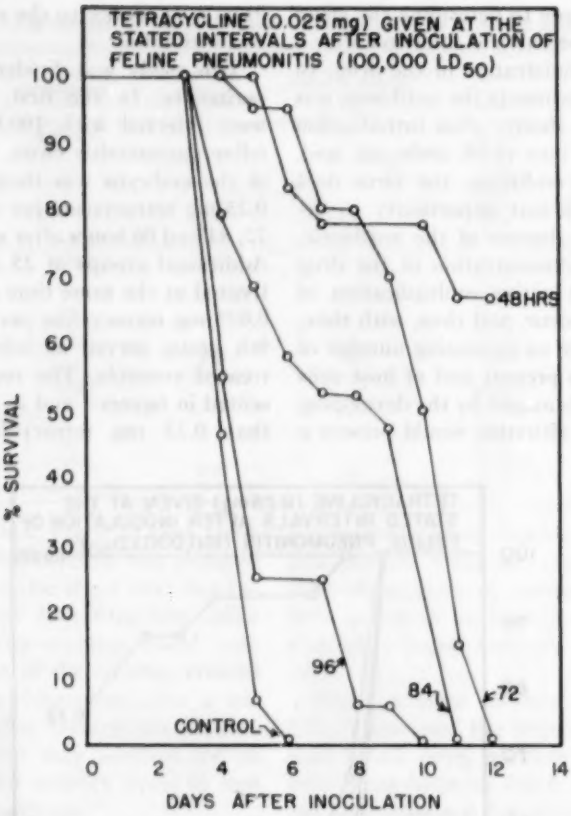

FIG. 2.—Effect of tetracycline (0.025 mg) given at the stated intervals of time after inoculation of feline pneumonitis virus (100,000 LD_{50}'s) into 6-day-old chick embryos.

considerable activity even when administration of the drug was delayed for several days after virus infection. When given 48 hours after infection, there was complete protection of embryos. If the drug was administered after 72 hours, there was protection of 77% of the embryos. After a delay of 84 hours, although there was a drop in the effectiveness of the antibiotic, 43% of the infected embryos survived. As late as 96 hours after infection, 12 hours before the expected death of the embryos, a slight degree of protection of infected embryos was observed. The results obtained, using 0.025 mg tetracycline, although not as striking, present a similar picture. When given 48 hours after virus, tetracycline protected 67% of the infected embryos. When its administration was delayed for 72, 84, and 96 hours, there was a delay in death of treated embryos of 5.4, 3.3, and 0.9 days, respectively. Tetracycline, at this concentration, showed some activity even when administered 12 hours before the expected death of the embryos.

In the second experiment 11 groups of 30 embryos were infected with 1000 LD_{50}'s of feline pneumonitis virus. A group of 30 of the embryos was then treated with 0.25 mg tetracycline per

embryo 2, 72, 96, 108, and 120 hours after infection. Additional groups of 30 embryos were treated at the same time intervals with 0.025 mg per embryo, while a final group of 30 embryos served as infected but untreated controls. The data are presented in figures 3 and 4. Controls died within 6.0 days of infection. Employing 0.25 mg tetracycline, it is evident that the antibiotic was highly effective in preventing the death of infected embryos even when administration of the drug was delayed for as much as 108 hours after infection. Its effectiveness decreased only gradually with an increase in time between infection and administration of the drug, and even within 1 day of death of the untreated controls tetracycline postponed the death of treated embryos by an additional day. At a level of 0.025 mg, tetracycline was most effective when administered 2 hours after viral infection. After a delay of 72 hours there was a moderate drop in activity (68% alive), and by 96 hours this concentration of antibiotic could only delay the death of infected embryos (3.7 days). There was still a slight degree of effectiveness (0.5 day delay), however, when

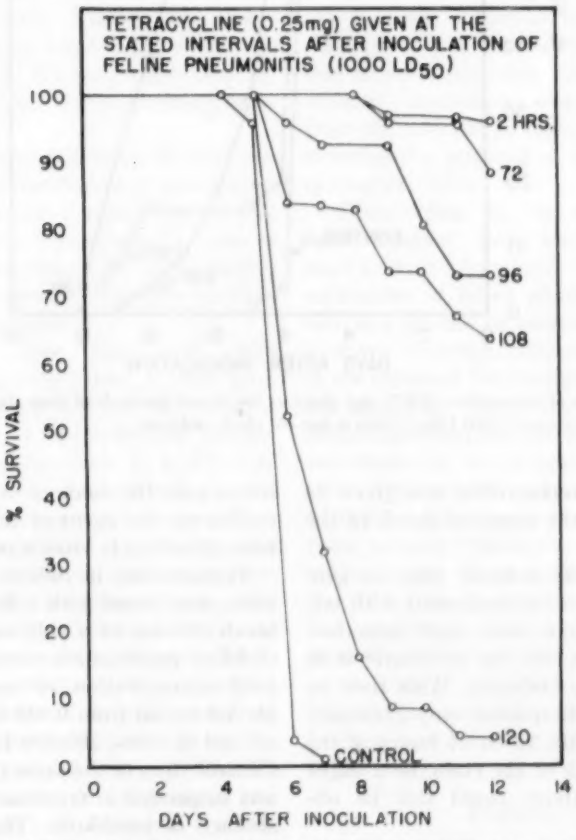

FIG. 3.—Effect of tetracycline (0.25 mg) given at the stated intervals of time after inoculation of feline pneumonitis virus (1000 LD_{50}'s) into 6-day-old chick embryos.

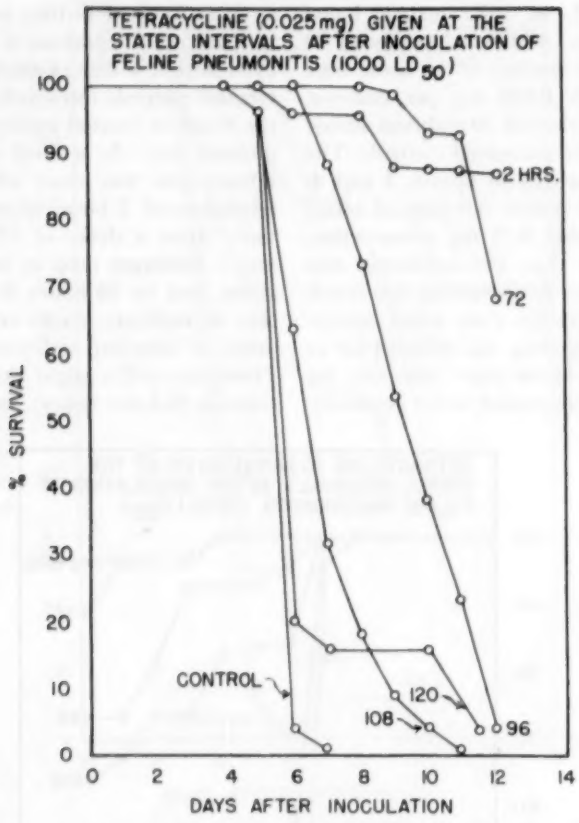

FIG. 4.—Effect of tetracycline (0.025 mg) given at the stated intervals of time after inoculation of feline pneumonitis virus (1000 LD_{50}'s) into 6-day-old chick embryos.

this level of tetracycline was given 24 hours before the expected death of the embryos.

These results indicate that, in general, the sooner the treatment with tetracycline beings after viral infection, the more effective the antibiotic is in protecting the embryos. With time its effectiveness diminishes only gradually and even within 12 to 24 hours of the expected death of the embryos a slight degree of activity could still be observed.

The activity of tetracycline on feline pneumonitis virus in vitro.—In order to investigate the mode of action of tetracycline on the agent of feline pneumonitis, its action in vitro was studied.

Tetracycline, in different concentrations, was mixed with a Bacto tryptose broth dilution of a yolk sac suspension of feline pneumonitis virus so that the final concentration of antibiotic employed varied from 0.025 to 2.5 mg per ml and of virus, 200,000 LD_{50}'s per ml. Control virus of the same concentration was suspended in tryptose broth in the absence of antibiotic. These mixtures were kept at room temperature and at 4 C. Aliquots of the former were taken

at 1 and 3 hours and of the latter at 3 and 48 hours for serial dilution in Bacto tryptose broth. One-half ml of appropriate dilutions was then injected into the yolk sac of chick embryos in order to determine the virus titer. A decrease in titer of the virus exposed to antibiotic to that of virus present in tryptose broth would indicate an in vitro action of tetracycline on feline pneumonitis virus.

It was found that tetracycline, like chlortetracycline,[3] has little or no action on feline pneumonitis in vitro. In no case was there a significant difference in titer between the antibiotic exposed and control virus. Tetracycline therefore must act on some stage in the development of feline pneumonitis in vivo and not on the inactive virus in vitro.

The persistence of virus in the chick embryos after administration of tetracycline.—Since tetracycline appears to have no activity on feline pneumonitis virus in vitro it was of interest to learn whether the drug possessed a virustatic or virucidal action in vivo.

Embryos were inoculated with 100,000 LD_{50}'s each and 1 hour after infection a group of 25 of the embryos received one of the following concentrations of tetracycline: 2, 1, 0.5, 0.25 mg. A 5th group of 25 embryos served as untreated but infected controls. Embryos were incubated for 12 days and all embryos surviving this period were examined for the presence of virus. The yolk sac of each embryo was harvested and impression smears prepared. These were stained, employing Macchiavello's technique. Pools of 4 yolk sacs from each group were emulsified with sand in a sterile mortar and pestle and a 10% suspension was made using equal volumes of sterile yolk fluid and tryptose broth as diluent. One-half ml of this suspension was then injected into the yolk sac of 6-day-old chick embryos to determine whether infective virus was present.

It was found that 95 to 100% of the treated embryos survived the incubation period, whereas controls died within 4 to 5 days after virus infection. Examination of stained preparations from surviving embryos revealed the presence of feline pneumonitis virus in every case. Furthermore, the virus was present largely as initial bodies and plaques; relatively few elementary bodies were in evidence. Heavy concentrations of elementary bodies were seen, however, in stained preparations from the controls. Embryos which received the 10% yolk sac suspension succumbed due to virus infection. Examination of stained impression smears prepared from the yolk sac of these embryos also revealed the presence of large numbers of viral particles.

Tetracycline, at the concentrations used, behaved more like a virustatic than a virucidal agent. It inhibited multiplication of feline pneumonitis virus but was unable to eliminate the virus in vivo. Although infected treated embryos survived the incubation period in all instances, virus, chiefly as initial bodies and plaques, was present in yolk sac tissue. In the presence of the drug feline pneumonitis is able to form these bodies but subsequent multiplication of these forms is inhibited to a very considerable extent. Since embryos receiving the 10% yolk sac suspension succumbed due to feline pneumonitis virus infection, this would indicate that the virus, in the absence of inhibitory concentrations of the drug, was able to multiply freely in vivo and bring about the death of the embryos.

SUMMARY

1. Tetracycline and oxytetracycline show equal but higher activity than chlor-

tetracycline in protecting chick embryos infected with feline pneumonitis virus. It is suggested that the greater instability of chlortetracycline in vivo may account for this difference. Tetracycline and oxytetracycline are as effective at 0.031 mg as at much higher concentrations of these drugs.

2. The effectiveness of tetracycline within wide limits is independent of the size of the virus inoculum employed.

3. Tetracycline possesses considerable activity in protecting embryos infected with feline pneumonitis virus even when there is a considerable delay between the time of infection and administration of the drug. The effectiveness of tetracycline decreases gradually with a delay in its administration. When administered 12 to 24 hours before the expected death of the embryos, the drug is still active.

4. Tetracycline is without activity on feline pneumonitis virus in vitro.

5. In vivo, tetracycline acts chiefly as a virustatic rather than as a virucidal agent. Initial bodies and plaques are formed in the presence of the antibiotic but subsequent multiplication of these bodies is inhibited to a considerable extent. Virus, once released from the presence of the antibiotic, is able to multiply freely once again and bring about the death of a susceptible host.

CELLULAR FACTORS IN ACQUIRED IMMUNITY TO *TRICHINELLA SPIRALIS*, AS INDICATED BY CORTISONE TREATMENT OF MICE

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In a previous report (Coker, 1956) it was demonstrated that cortisone treatment rendered immunized mice essentially nonimmune to *Trichinella spiralis* infection, and, in addition, seemed to prevent in these mice the gain of acquired immunity that nonimmunized mice ordinarily develop between 11 to 14 days following infection. Associated with these findings were those showing that the hormone had a demonstrable effect upon the lymphoid-macrophage system; it caused a reduction in spleen size, a lymphocytopenia and an eosinopenia.

A cellular response in the intestine of mice has been shown to be associated with immunity to *T. spiralis* (Larsh and Race, 1954). In view of this, and the results of the previous experiment of this series mentioned above, it seemed advisable to study directly the effect of cortisone upon the cellular response in the intestine and its relation to the operation of this immunity. Provided that cortisone produced a demonstrable depression of the cellular response in the intestine, this approach would afford an opportunity to test the hypothesis of Larsh (1953) that the operation of acquired immunity against *T. spiralis* in mice is dependent upon the "primary action(s) of specific antibodies with secondary cellular cooperation." The present experiment was designed, therefore, with the hope that further light might be shed on the mechanism of this immunity.

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MATERIALS AND METHODS

The source of the mice and the methods used in infecting them, the methods of preparing and injecting the cortisone,† making adult worm counts and analyzing the data statistically were approximately the same as indicated previously (Coker, 1955a). It is important to point out that for the present study a 0.5 mg dose of cortisone was used. It was given daily by the subcutaneous route beginning 3 days prior to the challenging infection and continuing until necropsy. For the histologic study, the entire anterior half of the small intestine of two mice of each group at each time interval was fixed in Helly's solution (Larsh and Race, 1954). Each portion was then cut into six small pieces which were sectioned together in tissue-mat at ten mc, stained with Ehrlich's haematoxylin and eosin-azur II, and mounted in balsam. The final preparations were studied under oil immersion.

EXPERIMENTAL PROCEDURES

Experimental design.—The purpose of this experiment was to determine the effect(s) of cortisone upon the cellular response in the intestine and its relation to the operation of immunity against *T. spiralis*.

† Cortisone acetate Merck ("Cortone") supplied by Merck and Company, Rahway, N. J., through the courtesy of Dr. Ashton C. Cuckler.

TABLE 1.—*Average adult worm counts from the small intestines of various groups of mice 7 and 14 days following a challenging infection with 400* T. spiralis *larvae given to groups II to IV. Groups I, III, and IV had been immunized.*

Group	Average adult worm counts	
	(a) 7 days	(b) 14 days
I Persistency controls (8 mice)	2.8	
II Nonimmune controls (16 mice)	344.0	196.1
III Immune controls (16 mice)	273.9	140.5
IV Cortisone experimentals (16 mice)	333.0	248.1

For the portion of the experiment dealing with effects on immunity, 56 mice were used (table 1). Fourteen of these were females, which were distributed equally among the various groups to be described. Sixteen mice were not immunized; the remaining 40 mice were immunized according to the same schedule and method use in the earlier experiment. That is, 8 weeks from birth they were given the first of 3 stimulating (immunizing) infections of 200 larvae; the 3 stimulating infections were spaced 21 days apart, followed by a 30-day period to allow for the elimination of the worms of the last stimulating infection before further treatment.

The 40 immunized mice and the 16 nonimmunized mice were divided into 4 main groups (I to IV) of 16 mice each (except group I, which contained 8 mice; see table 1). Each main group (except group I) was subdivided into two subgroups, (a) and (b), of 8 mice each, which were sacrificed for counts of adult worms in the small intestine at 7 days (a) and 14 days (b) following a challenging infection of 400 *T. spiralis* larvae. These various groups are now described briefly to make clear the design and purposes of the experiment.

The mice of group I (persistency controls) were immunized but not challenged. Any worms found in the small intestines of these mice would be those surviving from the stimulating infections. Since very few worms were found (table 1), it can be assumed that the worms recovered from the mice of the other groups were those developing from the challenging infection.

The mice of group II (nonimmunized controls) were not immunized but were challenged. Worm counts of this group would serve as a measure of the natural immunity of this strain of mice.

The mice of group III (immune controls) were immunized and challenged. Following the challenging infection, worm counts of this group, compared to those of group II, would indicate the level of acquired immunity established by the immunization procedure.

The mice of group IV (cortisone experimentals) were immunized, challenged and treated with cortisone. As mentioned above, these mice received a

TABLE 2.—*Student's* t *tests of the significances of the differences between the means of adult worm counts. The standard error (27.045) of the difference between the means was calculated using the pooled variances of all the groups, giving 42 degrees of freedom.*

Comparisons		t value	Significance level
No.	Type		
1	Immune_7 vs. nonimmune_7	2.59	0.02
2	Immune_{14} vs. nonimmune_{14}	2.06	0.05
3	Immune_7 vs. immune_{14}	4.93	<0.01
4	Nonimmune_7 vs. nonimmune_{14}	5.47	<0.01
5	Immune_7 vs. cortisone_7	2.19	0.05
6	Cortisone_7 vs. nonimmune_7	0.41	NS
7	Immune_{14} vs. cortisone_{14}	3.98	<0.01
8	Cortisone_{14} vs. nonimmune_{14}	1.92	NS
9	Cortisone_7 vs. cortisone_{14}	3.14	<0.01

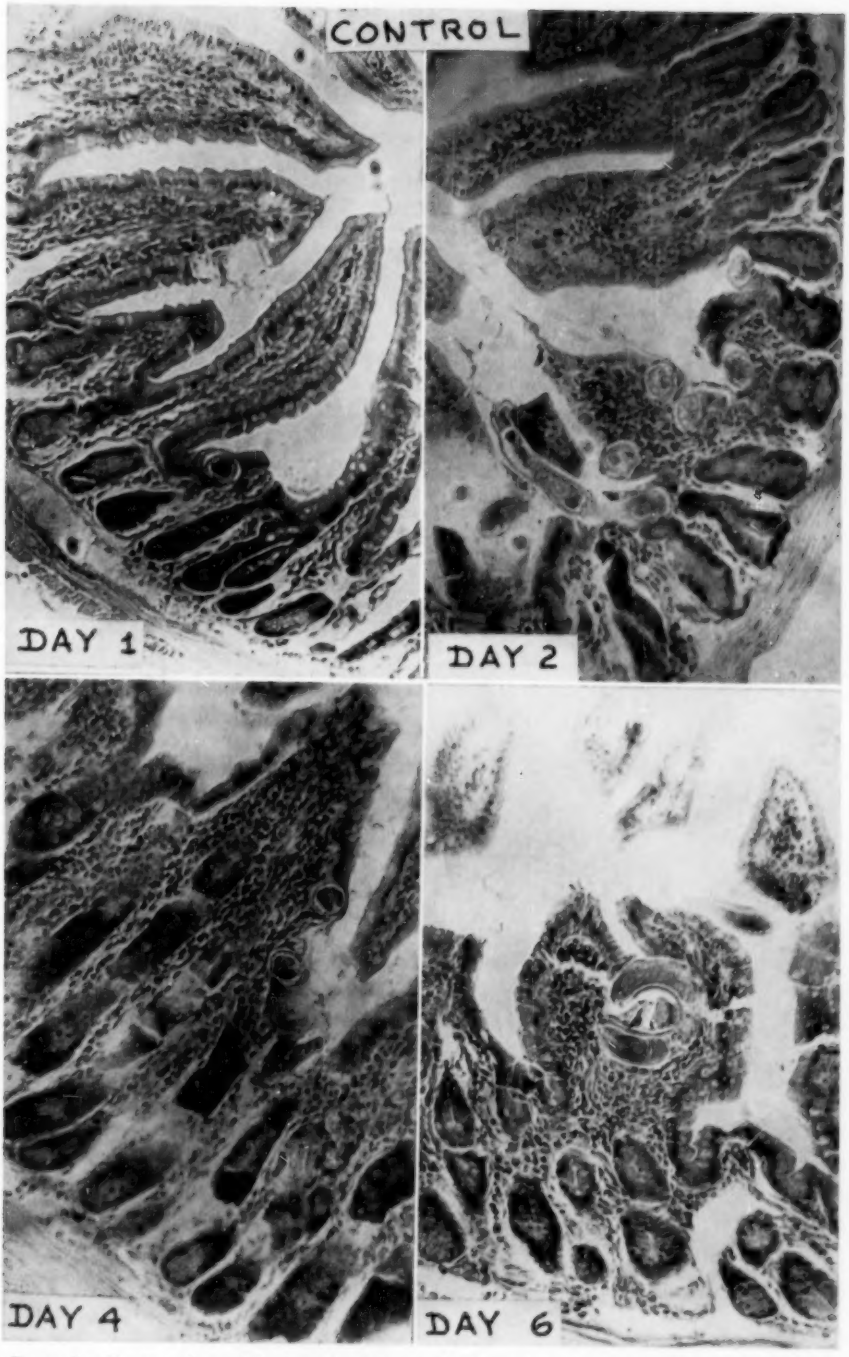

PLATE I.—Cross sections of the intestine of immunized control mice on days 1, 2, 4 and 6 following a challenging infection with 400 *T. spiralis* larvae. All photographs were taken at the same magnification, using a compound microscope with 10× ocular and 10× objective. The characteristic cellular reaction was first noticeable grossly on day 2, reached a peak on day 4, and began to subside to a chronic state on day 6. Detailed descriptions are given in the text.

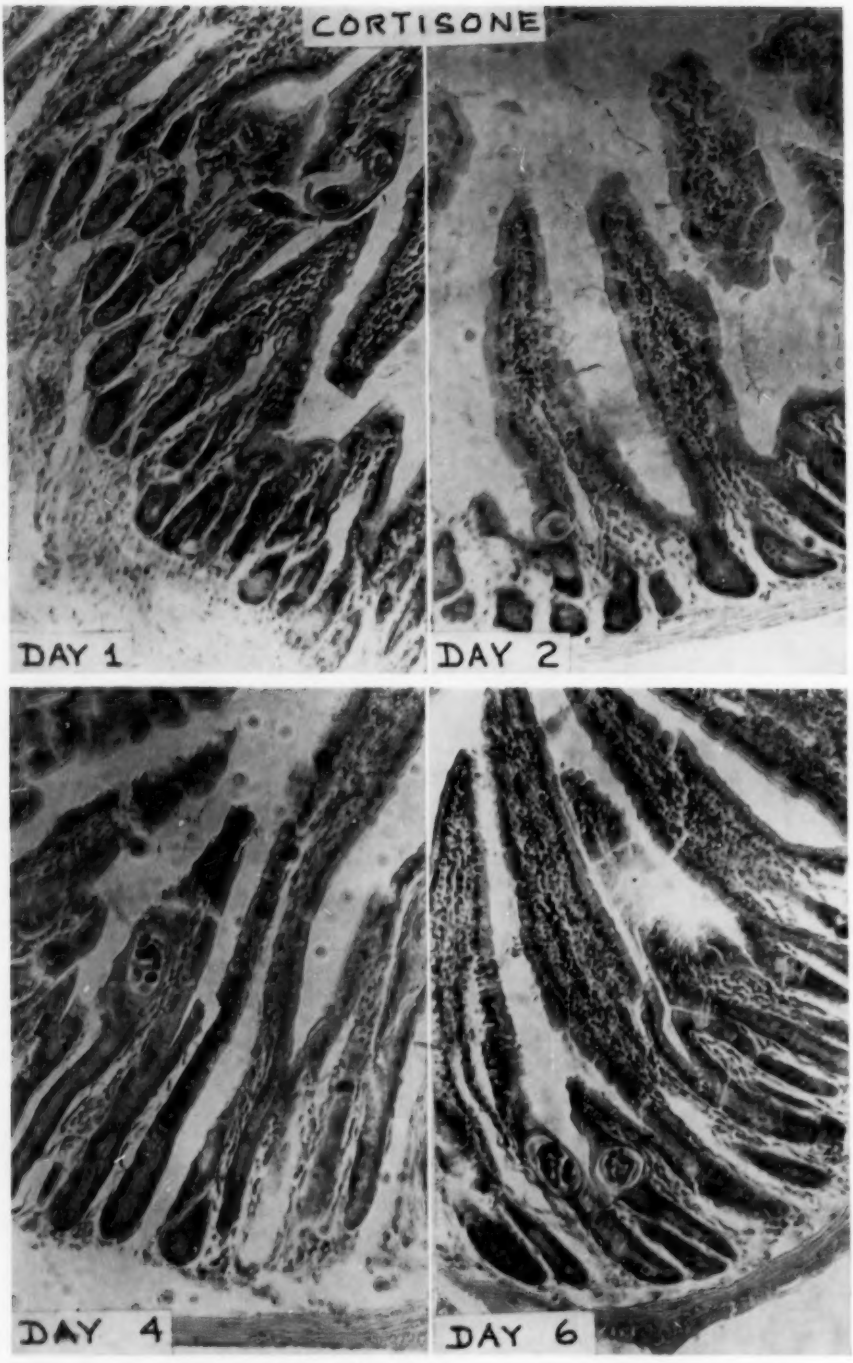

PLATE II.—Cross sections of the intestine of immunized cortisone-treated mice on days 1, 2, 4 and 6 following a challenging infection with 400 *T. spiralis* larvae. All photographs were taken at the same magnification, using a compound microscope with 10× ocular and 10× objective. The cortisone treatment completely suppressed the normal cellular reaction. Compare particularly the condition on day 4 with that shown for day 4 in the control mice (plate I). Detailed descriptions are given in the text.

daily subcutaneous injection of 0.5 mg of cortisone beginning 3 days prior to the challenging infection and continuing until necropsy. Worm counts from this group would indicate the effect of the cortisone treatment upon acquired immunity.

Since the maximum difference in worm counts between immunized and nonimmunized mice occurs 7 days after a challenging infection (Larsh et al, 1952), the 14-day necropsy period was not necessary for this portion of the experiment. However, it was included to test the earlier findings on the prolonged effect of cortisone (Coker, 1956).

For the cellular reaction study, 36 additional immunized male mice were set aside. Eighteen of these were comparable to group III mice (immune controls); the remaining 18 were treated with cortisone and were comparable to group IV mice (cortisone experimentals). Two mice was selected from each of the two groups at the following time intervals after the challenging infection with *T. spiralis*: 12 hours, days 1, 2, 3, 4, 5, 6, 7 and 9. The intestinal tissue for histologic study was prepared as described above.

EXPERIMENTAL RESULTS

Adult worm counts.—The mean worm counts of the various groups are presented in table 1 and the statistical analyses are shown in table 2. When the averages were compared one with another by means of Student's *t* test, they indicated that: (1) the immunization program was successful in establishing immunity (comparisons 1 and 2 in table 2); (2) the immune controls lost a significant number of worms between 7 and 14 days (comparison 3); (3) the nonimmune mice gained the customary acquired immunity between 7 and 14 days (comparison 4); (4) the cortisone treatment destroyed the established immunity as measured at 7 and 14 days following the challenging infection (comparisons 5 and 7); and (5) the cortisone experimentals had no greater immunity than the previously uninfected controls at 7 and 14 days (comparisons 6 and 8). It would appear from comparisons 8 and 9 that despite cortisone treatment there was loss of worms between 7 and 14 days. This will be considered further in the *Discussion*.

Histologic findings.—As expected, there were slight qualitative and quantitative differences in the inflammation noted in sections of different pieces of intestine sampled at the same period after the challenging infection. The variation in the number of eosinophils in the mice not receiving cortisone was particularly outstanding. However, by examining a large series of slides, it was not difficult to define the typical conditions for the various time periods. These are presented below.

The worms were found wholly or partly imbedded in the columnar mucous epithelium lining the villi. Occasionally one was observed which had pierced completely through several villi and was in intimate contact with the connective tissue stroma of the lamina propria. Photomicrographs of representative samples of the intestine of both groups of mice are shown in plates I and II, covering days 1, 2, 4 and 6 after the challenging infection with *T. spiralis*.

Immune control group (plate I).—At *12 hours* the composition of the lamina propria appeared normal. There were moderate numbers of mononucleate cells consisting of large and small lymphocytes, polyblast stages, large irregular cells resembling macrophages, occasional plasma cells and precursors. Eosinophils were not infrequent, occurring mainly in the lower lamina propria and in the submucosa. Neutrophils were rare to absent. Cross sections of blood vessels showed only an occasional leucocyte within them. On *day 1* the condition

was very similar to that at 12 hours, with the exception that a few neutrophils appeared and there was a tendency for some eosinophils to occur further out in the villi. On *day 2* inflammation was definitely in evidence. Moderate numbers of neutrophils appeared; they were more frequent in the submucosa and basal lamina propria, and rarer in distal parts of the villi. Some blood vessels contained an abundance of leucocytes, chiefly neutrophils, which in some instances appeared to be migrating through the blood vessel wall into the surrounding tissues. Mitotic figures were observed in some of the mononuclear cells. The total number of cells appeared to have increased over that of day 1. An occasional monocyte was seen. On *day 3* the inflammation was approximately the same in character as that of day 2, with a few more cells in evidence. Neutrophils were more widespread and abundant. On *day 4* the inflammation reached its peak. Neutrophils were common everywhere. Eosinophils were fairly abundant, extending well out into the villi. Blood vessels contained lymphocytes and neutrophils. The monocuclear cells were mostly plasmacytes, lymphocytes, a few macrophages and an occasional monocyte. Some of the mononuclears were in the process of dividing. On *day 5* the inflammation appeared to be subsiding. The number of neutrophils had declined considerably. Eosinophils were of normal occurrence. Most of the infiltrate was mononuclear, consisting mainly of plasmacytes, followed in abundance by lymphocytes and macrophages. Leucocytes were still observed in the blood vessels. On *day 6* conditions were similar to those noted on day 5. Some sections showed a striking abundance of eosinophils. On *day 7* the reaction continued to subside. Most of the cells, with the exception of a fair abundance of eosinophils, were mononuclears, mainly plasmacytes and lymphocytes with a few macrophages. There were very few neutrophils. On *day 9* the inflammation appeared to be at a chronic level equivalent to day seven. Eosinophils were abundant and widely dispersed throughout the entire mucosa and submucosa. Blood vessels rarely showed leucocytes.

Cortisone-treated experimental group (*plate II*).—At *12 hours* the cells of the lamina propria were almost entirely mononuclears; plasmacytes were the most abundant type, with a few small lymphocytes and some polyblasts. Neutrophils and eosinophils were rare to absent. The cells in general were more sparsely distributed than in the controls. On *day 1* the condition was approximately comparable to that at 12 hours. On *day 2* eosinophils continued to be rare. Some neutrophils were present but they were uncommon and occurred chiefly in the submucosa. Macrophages and polyblasts were seen only rarely, apparently having been replaced by plasmacytes, some of which had bizarre shapes. Lacteals tended to occupy proportionately more space in the lamina propria. The supporting framework of connective tissue appeared to be somewhat reduced in comparison with that observed in the controls. Some of the blood vessels showed an occasional leucocyte. On *day 3* both eosinophils and neutrophils were rare. The cell population in the lamina propria and submucosa was sparse, appearing to be mainly plasmacytes, unrecognizable types of mononuclears, and the supporting framework of fibroblasts and other fibrous and elastic tissue. The villi tended to be long and thin and filled with blood. Lacteals were prominent. On *day 4* the villi, in many cases, were almost barren of enclosed cells other than the native supporting stroma of fibroblasts, other connective tissue, and extensions of the muscularis mucosa. Both eosinophils and neutrophils were rare. Some sec-

tions showed a less drastic effect, but the contrast with the cellular effusion noted in the controls was very striking. Blood was noticeable in the villi. On *days 5, 6, 7, and 9* there was little change from *day* 4.

Other observations.—The occurrence of eosinophils was, for the most part, independent of the time interval after infection. The cellular infiltration in both groups did not differ in the areas immediately adjacent to the worms and in other comparable areas of the mucosa.

DISCUSSION

In this experiment, as in an earlier one (Coker, 1956), cortisone given to mice was shown to eliminate established immunity to *T. spiralis*, according to the accepted criterion of adult worm counts at seven days after a challenging infection. There was, however, one difference in the results of these two experiments. Statistical analysis of the results of the previous experiment indicated that, while fewer worms were present at 14 days than at 7 days in the cortisone-treated immunized mice, the difference in counts was not significant. This suggested that immunity was eradicated for at least 14 days. In the present experiment the counts were significantly lower at 14 days. Therefore, it may be open to question as to whether or not cortisone, in the dosage used, destroyed immunity for as long as 14 days. It would appear that there was a relatively small, but real, loss of worms after 7 days.

A cellular reaction was shown to develop in the small intestine of the immunized mice after they had been challenged with worm larvae. The nature and timing of this reaction were similar in all major respects to that described by Larsh and Race (1954) in immunized mice. First, the inflammation began its development as early as the 1st day after infection, progressed to a peak on the 4th day, and then subsided into a chronic state persisting into the ninth day when observations were stopped. Second, the predominant cells in the early, acute phase were neutrophils, which were accompanied, and later supplanted, by the mononuclear series. A minor difference from the earlier work was observed in that eosinophils seemed to play some part in the reaction. They tended to become more widely dispersed throughout the lamina propria as the reaction developed, and frequently were very abundant. This apparent discrepancy from the earlier findings is probably based upon a different staining technique.

The experiment established clearly that the cellular response in the intestine was almost completely prevented in the mice treated with cortisone. Intestinal eosinophils, normally frequent to abundant, practically disappeared. The invasion of neutrophils, which are characteristic members of the acute cellular response, failed to develop, since only a few widely scattered cells were observed. Likewise, the native cell population in the lamina propria and submucosa of the small intestine of the cortisone-treated mice was, on the whole, greatly reduced as compared with that of the controls.

These findings have a direct bearing on the hypothesis that acquired immunity to *T. spiralis* in mice is due to the primary action(s) of specific antibodies with secondary cellular cooperation. This hypothesis was prepared by Larsh (1953) after making studies in old mice to test directly the two major views (local and general) as to the nature of this immunity. Before discussing the relation of the present findings to this hypothesis, it is advisable to review briefly some of the immediately related foundations upon which it rests.

That there is a protective factor(s) in the blood serum of immunized animals

was indicated directly by Mauss (1940), who found that *T. spiralis* larvae were significantly reduced in infectivity when incubated in antiserum, and by Oliver-Gonzalez (1940), who found that both larval and adult worms died sooner (with precipitates about their body openings) in antiserum than in normal serum. This protective factor(s) could well be comprised, at least partially, of specific antibody, since Hendricks (1950, 1952) found an inverse relationship between the titer of specifically aroused circulating precipitins and the number of adult worms recovered after a challenging infection in mice given varying numbers of stimulating infections. The specific nature of the anti-trichinella antibody has been demonstrated frequently. This factor is not produced unless the host has been stimulated to produce it by infection with the parasite or by inoculation with portions, extracts, or excretions and secretions of the parasite (Culbertson and Kaplan, 1938; Culbertson, 1942; Campbell, 1955; and others). Antiserum transferred to nonimmunized mice confers a degree of passive immunity (Culbertson and Kaplan, 1938; Hendricks, 1953).

There is less, but nevertheless strong, evidence that cellular reactions are participants in the immunity mechanism against *T. spiralis*. As already indicated, Larsh and Race (1954) showed that an acute cellular reaction reached a peak in the small intestine of immunized mice shortly before a significant loss of worms occurred in similar mice. They also showed a comparable relationship in mice following an initial infection. Stoner and Hale (1952) found that whole body irradiation of mice with Cobalt ^{60} gamma rays not only increased the susceptibility to trichinella but also decreased the number of circulating leucocytes. Cox (1952) suggested that increased natural and acquired immunities of mice to *T. spiralis*, following prior oral infection with *Ancylostoma caninum* larvae, was due to the intestinal inflammation induced by *A. caninum*.

The results of the present experiment offer further strong evidence that the cellular reaction is a feature of the immunity mechanism. In fact, there is evidence that it may be of paramount importance in causing the elimination of the worms. Cortisone treatment of the mice eliminated both the cellular reaction and the 7-day immunity of the mice. Another experiment has been performed (Coker, 1954) to test whether cortisone treatment destroyed the serum protective factor(s) as well. In this experiment serum was taken from cortisone-treated immunized mice and injected into nonimmunized mice, which were then given an infection. Worm counts of these mice were significantly lower than those of controls, suggesting that the serum protective factor(s) was not seriously inhibited or destroyed in the immunized mice treated with cortisone. This observation is not inconsistent with those of other workers showing that under some circumstances measurable serum antibody concentrations are not affected, or become elevated above normal, following treatment of the experimental animal with ACE or cortisone (Dougherty et al, 1945; Halper et al, 1952; Hammond and Novak, 1950).

If, for the moment, it is assumed that cortisone did not destroy the serum protective factor(s) in the mice of the present experiment, then it can be accepted that the serum protective factor(s) alone was not effective in eliminating the worms. It seems obvious that this factor(s) requires assistance to bring about the expulsion of the worms, at least when the worm burden is at the level reported here and in earlier studies on this immunity. Previous work, cited above, and the present findings suggest

that the cellular reaction constitutes this required assistance. This is not to imply that the serum protective factor(s) does not function in the absence of a normal cellular response. It is likely, as expressed by Larsh and Race (1954), that such a factor(s) can have a direct effect upon the worms—for example, effects upon their development or upon their "desire" to remain in the environment. Indeed, some sort of direct effect upon the worms may have accounted for the partial loss of worms between 7 and 14 days in the cortisone-treated mice of the present experiment. However, it would seem that when inflammation is firmly suppressed, as by cortisone, the worm elimination process is unable to function perfectly and the worms persist in large numbers.

There is further indirect evidence for the importance of the cellular reaction against trichinella. Baughn (1952) found that adrenalectomy of mice increased their natural and acquired immunities to *T. spiralis*. Adrenalectomy results in many physiologic disturbances, but one of its effects in mice results in an increase in the amount of lymphatic tissue, the effect being maximal at five days (Santisteban, 1951). Most of Baughn's adrenalectomized mice were challenged 4 days after adrenalectomy, at a time when, presumably, their lymphatic reserves were approaching a maximum. He found that the precipitin titer of the adrenalectomized, immunized mice was no different from that of the sham-operated controls or from that of the non-operated immunized controls. There would seem to be a close relationship between the findings of Baughn, Santisteban, a host of other workers and the present investigator. It may be visualized by regarding two extremes. At one extreme lies an association of a decrease in cortisone (implied in adrenalectomy), an increase over normal in lymphoid reserves, and an increase over normal in either natural or acquired immunity to trichinella. At the other extreme lies another association of an excess of cortisone, a decrease in lymphoid reserves, and a decrease in acquired immunity to trichinella. Within this framework the serum protective factor(s) undoubtedly operates, in ways previously discussed, but its effectiveness in inducing an expression of immunity is not likely to be independent of other factors.

Evidence that cellular reactions can be important regulators of other parasitic worm infections is provided by the work of Taliaferro and Sarles (1939 and 1942), Lindquist (1950) and Weinstein (1955). Taliaferro and Sarles demonstrated that, in immune rats, *Nippostrongylus muris* larvae tended to be immobilized and stunted in further development through antibody action, and then attacked by cellular elements, which often were able to encapsulate and destroy the immobilized worms. Lindquist found that *N. muris* was unable to complete its development in certain abnormal hosts. Instead, many of the worms were attacked, encapsulated and destroyed in the tissues, apparently without the action(s) of antibody. Weinstein administered cortisone to rats during and after immunization to *N. muris*. There was a striking reduction in the customary cellular response of the skin to a challenging infection with larval worms, and the worms were more successful in continuing their migration and development.

Both Larsh and Race (1954) and the present investigator noted that the cellular reaction in response to trichinella in mice is a general one throughout the mucosa. The infiltrating cells do not seek out and encapsulate the worms, at least within the time limits that the reaction has been observed. This raises the question as to the manner in which the cells are able to operate against the

worms to bring about their expulsion. The answer may be related to the fact that the various types of cells in an inflammatory exudate have capabilities in addition to those of encapsulation and phagocytosis (Taliaferro, 1949). For example, lymphocytes, neutrophils and macrophages can secrete ferments, which may have an adverse effect upon an invading organism. Eosinophils, which are prominent in chronic inflammation and are characteristic of parasitic infections, are believed to absorb and thus neutralize protein disintegration products such as can be expected to result from a foreign invasion of the tissue (Downey, 1938). Therefore, it would not be unreasonable to assume that the presence of the inflammatory cells and their secretion products, particularly in the intestinal area, would create an unfavorable environment for trichinella.

The evidence discussed above is quite suggestive that cellular reactions can be extremely potent participants in immunity mechanisms against certain worm parasites and against trichinella in particular. These reactions certainly do not occur without an appropriate stimulus. In some parasitic infections they are elicited very rapidly, as when *Nippostrongylus muris* attacks an abnormal host. On the other hand, in infections of nonimmunized mice with trichinella, a cellular reaction can not be demonstrated for several days after the larval infection, although the worms are undoubtedly secreting metabolic products from the moment of their establishment in the small intestine. Therefore, the secretions and excretions of these worms do not arouse an immediate cellular response that can be observed by the usual methods. Taliaferro and Sarles (1939) concluded that the reaction products of specific antibodies with the antigenic materials of *N. muris* served as the stimulus in arousing the specific cellular reaction against the worms. Larsh and Race (1954) have provided a similar explanation for the specific calling forth of the cellular response against *T. spiralis* in mice. According to available information, this concept would seem to merit careful consideration. It may be thought of as operating in three stages: (1) specific antibodies are produced against trichinella, the protective ones, no doubt, being formed by the antigenic stimulation of its excretions and secretions; (2) these antibodies react with the worms and/or their antigenic byproducts; and (3) the products of this antigen-antibody reaction act as an irritating stimulus for the characteristic inflammatory response.

The results of the present experiment are in harmony with the hypothesis (Larsh, 1953) that the immunity mechanism against trichinella in mice is due to the primary action(s) of specific antibodies with secondary cellular cooperation. While the action(s) of antibodies is, no doubt, the primary one, i.e., first to enter the protective reaction, the present results would seem to indicate that the cellular factor(s) is most important in bringing about the expulsion of the adult worms from the intestine.

SUMMARY

Cortisone administered to mice immunized against *Trichinella spiralis* caused: (1) an elimination of established immunity as judged by adult worm counts from the small intestine of various groups of mice 7 days after a challenging infection with larval worms, and (2) a firm suppression, for at least 9 days following the challenging infection, of the characteristic cellular response shown for immune controls.

The results of the experiment, when considered in the light of additional evidence drawn from the literature, are in harmony with the hypothesis that the immunity mechanism against this para-

site in mice is due to the primary action(s) of specific antibodies with secondary cellular cooperation. Moreover, the results seem to indicate that the cellular factor is of greater importance than the serologic factor(s) in bringing about the actual elimination of the worms from the small intestine. A possible mechanism for this postulated effect of the cellular reaction is discussed.

REFERENCES

Baughn, C. O. 1952, The effect of adrenalectomy on natural and acquired resistance of mice to *Trichinella spiralis*. J. Elisha Mitchell Sc. Soc. **68:** 207–221.

Campbell, C. H. 1955, The antigenic role of the excretions and secretions of *Trichinella spiralis* in the production of immunity in mice. J. Parasitol. **41:** 484–491.

Coker, C. M. 1954, Effects of cortisone on established immunity to *Trichinella spiralis* in mice, with reference to the role of the cellular factor(s) in the immunity mechanism. (Doctoral thesis) University of North Carolina, Chapel Hill, N. C.

———. 1955, Effects of cortisone on *Trichinella spiralis* infections in non-immunized mice. J. Parasitol. **41:** 498–504.

———. 1956, Some effects of cortisone in mice with acquired immunity to *Trichinella spiralis*. J. Infect. Dis. **98:** 39–44.

Cox, H. W. 1952, The effect of concurrent infection with the dog hookworm, *Ancylostoma caninum*, on the natural and acquired resistance of mice to *Trichinella spiralis*. J. Elisha Mitchell Sc. Soc. **68:** 222–235.

Culbertson, J. T. 1942, Active immunity in mice against *Trichinella spiralis* larvae. J. Parasitol. **28:** 197–202.

Culbertson, J. T. and Kaplan, S. S. 1938, A study upon passive immunity in experimental trichiniasis. Parasitology, **30:** 156–166.

Dougherty, T. F., Chase, J. H. and White, A. 1945, Pituitary-adrenal cortical control of antibody release from lymphocytes. An explanation of the anamnestic response. Proc. Soc. Exper. Biol. & Med. **58:** 135–140.

Downey, H. 1938, Handbook of Hematology. New York, Hoeber.

Halpern, B. N., Mauric, G., Holtzer, A. and Briot, M. 1952, Effect of cortisone upon the rise of serum antibody level after injection of a saturating dose of antigen in rabbits sensitized to ovalbumin. J. Allergy, **23:** 303–309.

Hammond, C. W. and Novak, M. 1950, Relation of adrenal cortical steroids to antibody release. Proc. Soc. Exper. Biol. & Med. **74:** 155–161.

Hendricks, J. R. 1950, The relationship between precipitin titer and number of *Trichinella spiralis* in the intestinal tract of mice following test infections. J. Immunol. **36:** 173–177.

———. 1952. Studies in mice on the dual antibody basis of acquired resistance to *Trichinella spiralis*. J. Elisha Mitchell Sc. Soc. **68:** 12–26.

———. 1953, Passive transfer of resistance to *Trichinella spiralis* in mice. J. Parasitol. (Aug. suppl.) **39:** 40.

Larsh, J. E., Jr. 1953, Studies in old mice to test the hypotheses of local and general immunity to *Trichinella spiralis*. J. Infect. Dis. **93:** 282–293.

Larsh, J. E., Jr., Gilchrist, H. B. and Greenberg, B. G. 1952, A study of the distribution and longevity of adult *Trichinella spiralis* in immunized and non-immunized mice. J. Elisha Mitchell Sc. Soc. **68:** 1–11.

Larsh, J. E., Jr. and Race, G. J. 1954, A histopathologic study of the anterior small intestine of immunized and non-immunized mice infected with *Trichinella spiralis*. J. Infect. Dis. **94:** 262–272.

Lindquist, W. D. 1950, Some abnormal host relationships of a rat nematode, *Nippostrongylus muris*. Am. J. Hyg. **52:** 22–41.

Mauss, E. A. 1940, The *in vitro* effect of immune serum upon *Trichinella spiralis* larvae. Am. J. Hyg. **32-D:** 80–83.

Oliver-Gonzalez, J. 1940, The *in vitro* action of immune serum on the larvae and adults of *Trichinella spiralis*. J. Infect. Dis. **67:** 292–300.

Santisteban, G. A. 1951, The growth and involution of lymphatic tissue and their relation to secretions of the adrenal cortex in CBA mice. (Thesis) University of Utah.

Stoner, R. D. and Hale, W. M. 1952, The effect of cobalt^{60} gamma radiation on susceptibility and immunity to trichinosis. Proc. Soc. Exper. Biol. & Med. **80:** 510–512.

Taliaferro, W. H. 1949. The cellular basis of immunity. Ann. Rev. Microbiol. 1949: 159–194.

Taliaferro, W. H. and Sarles, M. P. 1939, The cellular reactions in the skin, lungs, and intestine of normal and immune rats after infection with *Nippostrongylus muris*. J. Infect. Dis. **64:** 157–192.

———. 1942, The histopathology of the skin, lungs, and intestine of rats during passive immunity to *Nippostrongylus muris*. J. Infect. Dis. **71:** 69–82.

Weinstein, P. P. 1955, The effect of cortisone on the immune response of the white rat to *Nippostrongylus muris*. Am. J. Trop. Med. & Hyg. **4:** 61–74.

DYNAMICS OF BACTERIAL INFECTIONS IN MICE UNDER CONDITIONS KNOWN TO ALTER SURVIVAL TIME

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The duration of a lethal infection in mice is known to vary under different experimental conditions. Massive dosages of bacteria (Olitzki, 1948), postinfection injections of metabolic inhibitors and intermediates (Berry and Mitchell, 1953b and c; Berry, Merritt, and Mitchell, 1954), and adjuvants such as mucin (Nungester, 1936) are known to alter survival time. It was shown previously (Berry, 1955) that postinfection injections of metabolic inhibitors and intermediates reduce the time required for a relatively constant lethal population of viable pathogens to accumulate within the body of the host. The alternative explanation, the possibility that sublethal amounts of such compounds increase the sensitivity of the experimental animals to the infectious agent, thereby decreasing the number of pathogenic cells required to kill, seems, therefore, unlikely. In view of this fact the question arises as to whether the lethal population of injected pathogenic organisms culturable at death of the mouse is relatively constant under other experimental conditions known to alter the duration of a fatal infection. In the experiments described in this report, the number of viable *Salmonella typhimurium* 1 and 6 hours postinfection and immediately after death has been determined in the bodies of control and immunized mice, in mice infected with mucin as an adjuvant, in mice acclimatized to simulated altitude, and in mice given a large number of heat-killed pathogens concurrently with the infectious dose. Results indicate that in these experimental groups a relatively constant population of viable pathogens is reached at the time of death.

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METHOD

The number of viable *S. typhimurium* in mice previously infected intraperitoneally with a saline suspension of the organism was determined by the method already described (Berry, 1955). Briefly, this involved either sacrificing the mouse by a blow on the head at a given time postinfection, or observing the animal at the time of death, skinning it, removing the feet and tail and weighing the carcass to the nearest quartergram on a triple-beam balance. The carcass was then placed in a Waring blendor and 9 ml of 0.86% solution of sodium chloride per gram of body weight were added. After blending for 5 minutes the homogenate was serially diluted with saline. From the final dilution, 0.1 ml was transferred to each of three SS agar (Difco) plates which were incubated at 37 C for 48 hours. The average number of colonies per plate multiplied by the dilution factor gave the number of *S. typhimurium* per mouse.

Two strains of *S. typhimurium* were used in these studies. The V31 strain was obtained from Dr. Howard A. Schneider, Rockefeller Institute, and the Texas strain, the one employed in previous studies reported from this laboratory, was originally isolated from an animal dead of mouse typhoid at the School Aviation Medicine by Dr. Roland B. Mitchell.

Heat-killed vaccines were prepared by culturing the organisms on nutrient agar (Difco) slants, washing the slants with saline, centrifuging the suspension and then incubating it in a water bath at 60 C for ½ hour. The suspension was checked for viability by subculturing in nutrient broth. Two percent formalin was added and the density of the suspension was adjusted to approximately one billion cells per cc, based on a dilution count of the

suspension that was made prior to heating. Mice were immunized by injecting intraperitoneally one-half billion cells of the vaccine 1 week before they were to be subjected to experimental procedures.

The culture used for infecting mice was grown in trypticase soy broth (BBL), transferring twice at 24-hour intervals, and once 15 hours before infection. The culture was then diluted with 0.86% saline and the mice were injected intraperitoneally with 0.5 ml of the final saline suspension. Dilution counts were made on each 15-hour culture employed.

The mucin suspension was prepared by autoclaving 5% gastric mucin (Winthrop-Stearns) in 0.86% saline at 15 pounds pressure for 20 minutes. This solution was used as the final diluent for the 15-hour culture with which the mice were infected.

The number of viable *S. typhimurium* in the bodies of artificially infected mice 1 and 6 hours postinfection and immediately after death was determined in the following groups of animals: mice infected with 50 to 80 million cells, strain V31; immunized mice infected with 50 to 80 million cells, strain V31; mice infected with 5 to 8 million cells, strain V31; mice infected with 5 to 8 million cells of strain V31 suspended in mucin; mice infected with 1 million cells of strain Texas and 1 and 6 hour counts on immunized mice infected with 1 million cells of strain Texas.

Death counts alone were made in the following additional groups: mice infected with 1 million or 200,000 cells of strain V31; mice infected with 1 million cells of strain V31 at the same time that either ½ billion or ½ billion cells of the heat-killed vaccine were administered; mice infected with 10 million cells of strain Texas; immunized mice infected with 10 million cells of strain Texas; and mice exposed to simulated altitude of 20,000 ft according to the technique described by Berry and Mitchell (1953a) for 3 weeks prior to infection and infected with 1 million cells of strain Texas.

Counts were made 30 hours postinfection on immunized mice infected with 50 to 80 million cells of *S. typhimurium*, strain V31.

Young adult female mice (and a few males) of the CF 1 strain from 2 to 4 months of age, and from 14 to 20 g in weight were used in these studies. They were housed in small galvanized iron cages with wood shavings for bedding. Their diet consisted of Purina dog chow checkers. Food and water were available to them at all times.

RESULTS

1. The number of S. typhimurium *in mice 1 hour postinfection*

All opaque, black-centered colonies, or colonies giving a yellow zone on the SS agar were assumed to be *S. typhimurium*. The potential errors involved in this procedure have been discussed by Berry, 1955.

The number of *S. typhimurium*, strain V31, in each of 13 control mice infected with 50 to 80 million cells is listed in the first column of table 1. Individual values range from a minimum of 219,000 to a maximum of 1,640,000 bacteria per gram of body weight, with an average of 640,000. The

TABLE 1.—*Estimated number of* S. typhimurium *per gram of body weight 1 hour postinfection in control mice, immunized mice, and mice injected with mucin as adjuvant. Each entry is the result obtained with individual animals.*

Number of *S. typhimurium* ($\times 10^6$) found 1 hour postinfection in mice infected with:					
Strain V31 at dosage level of:				Strain Texas at dosage level of:	
50 to 80 $\times 10^6$ in control mice	50 to 80 $\times 10^6$ in immune mice	5 to 8 $\times 10^6$ in control mice	5 to 8 $\times 10^6$ with mucin in mice	1 $\times 10^6$ in control mice	1 $\times 10^6$ in immune mice
219	21	30	24*	0.55	0.48
280	32	32	37*	0.87	0.52
410	33*	45	39*	0.99	0.90
470	59*	62	40	2.1	1.1
480	77*	87	41	2.3	1.7
550	104*	100	44	2.6	2.3
561	107	132	73	2.9	2.7
582	133*	139		3.2	2.8
670	153			3.4	2.9
780	260*			4.1	5.9
790	603				6.1
830					8.0
1640					
Average 640	144	78	43	2.3	2.8

* Male mice.

average count per gram of body weight is approximately 1/15 the average total count per mouse. Thus it can be seen that during the initial hour of infection about four-fifths (40 million/50 million) of the injected bacteria are rendered nonviable in the mouse.

Results obtained with each of 11 immunized mice given the same infectious dose are listed in column 2 of table 1. The range of values is from 21,000 to 603,000 bacteria per gram of body weight, with the average, 144,000. The large range of values in this group of mice is perhaps attributable, in part, to varying degrees of immunity conferred on the mice.

Thus, the average number of bacteria per gram of body weight 1 hour postinfection is approximately five times greater in control mice than in immunized mice. According to the rank order test of White (1952), this difference would occur by chance less than one time in a thousand. It is evident, therefore, that fewer bacteria remain viable in immunized than in control mice.

The values obtained with 8 control mice infected with 5 to 8 million cells of strain V31 are listed in column 3 of table 1. The counts per gram of body weight range from 30,000 to 139,000 and average 78,000. Again, approximately four-fifths of the bacteria lost viability during the initial hour of infection, despite the fact that in this group of animals the infectious dosage was only one-tenth that given the control group infected with 50 to 80 million cells.

Bacterial counts obtained from 7 mice infected with 5 to 8 million cells suspended in mucin are to be found in column 4 of table 1. The range of values is from 24,000 to 73,000 cells per gram of body weight. Although the average (43,000) is almost one-half that obtained in control mice (78,000), the rank order test shows a high probability that the difference is attributable to chance alone.

The number of *S. typhimurium*, strain Texas, in each of 10 control mice infected with 1 million cells and sacrificed 1 hour postinfection is listed in column 5 of table 1. Individual values range from a minimum of 550 to a maximum of 4100, with an average of 2300 bacteria per gram of body weight. Approximately nine-tenths of the injected bacteria failed to grow after the initial hour of infection.

Results obtained with 12 immunized mice given the same infectious dose of strain Texas as the control mice are found in the last column of table 1. The counts range from 480 to 8000; the average is 2800 bacteria per gram of body weight. According to the rank order test there is no significant difference between 1 hour counts made on control and immunized mice infected with 1 million cells of the Texas strain. This differs from the results obtained when the larger dosage of strain V31 was used (see above) and suggests that with smaller infectious dosages the immune mechanisms offer no evident advantage within this period of time.

2. *The number of* S. typhimurium *in the body of mice 6 hours postinfection*

In the first column of table 2 is listed the number of *S. typhimurium*, strain V31, 6 hours postinfection in 7 control mice infected with 50 to 80 million cells. The smallest value is 9,200,000; the largest is 46,000,000; and the average is 24,000,000 bacteria per gram of body weight. Thus it is evident that in the control mice there is a very large increase in the bacterial population between 1 and 6 hours postinfection (compare column 1, table 1 with column 1, table 2).

Counts obtained from 13 immunized

TABLE 2.—*Estimated number of* S. typhimurium *per gram of body weight 6 hours postinfection in control mice, immunized mice, and mice injected with mucin as adjuvant. Each entry is the result obtained with individual animals.*

Number of *S. typhimurium* ($\times 10^6$) found 6 hours postinfection in mice infected with:					
Strain V31 at dosage level of:				Strain Texas at dosage level of:	
50 to 80 $\times 10^6$ in control mice	50 to 80 $\times 10^6$ in immune mice	5 to 8 $\times 10^6$ in control mice	5 to 8 $\times 10^6$ with mucin in mice	1 $\times 10^6$ in control mice	1 $\times 10^6$ in immune mice
9,200	113	45	1,900*	15	0.13
12,000	140	124	4,000*	26	0.31
12,000	160*	226	4,500*	47	0.31
19,000	160	251	7,200*	55	0.43
23,000	190	294	8,000*	56	0.91*
45,000	350	420	11,000*	81	2.0*
46,000	510*	459		82	2.4*
	535*	604		85	2.6
	540	754		157	2.7
	600			160	
	830			490	
	1270*				
	1930*				
Average 24,000	563	353	6,100	114	1.3

* Male mice.

mice given the same infectious dosage as the control animals are listed in column 2 of table 2. The number of *S. typhimurium* per gram of body weight varies from a minimum of 113,000 to a maximum of 1,930,000, with an average of 560,000. Although there is a significant increase ($P=0.001$) in the bacterial population in immunized mice between 1 and 6 hours postinfection (compare data of tables 1 and 2), this increase is not nearly so great as that found in the control mice (column 1 vs. column 2, table 2).

Values obtained from 9 control mice infected with 5 to 8 million cells of *S. typhimurium*, strain V31, are to be found in column 3 of table 2. They range from 45,000 to 754,000, with an average of 353,000 bacteria per gram of body weight. Counts obtained with 6 mice infected with 5 to 8 million cells, strain V31, using mucin as an adjuvant (column 4, table 2) range from a minimum of 1,900,000 to a maximum of 11,000,000, with an average of 6,100,000. It is evident that there is a great difference between the 6 hour counts obtained from the control mice and those given mucin, and the probability of this occurring by chance is less than 0.001 by the rank order test. There is an even more striking difference between the 1 and 6 hour counts obtained with mice injected with a mucin suspension of the bacteria (compare column 4, table 1 vs. column 4, table 2).

The number of *S. typhimurium*, strain Texas, found 6 hours postinfection in 11 control mice infected with 1 million cells is listed in table 2, column 5. The bacterial count per gram of body weight ranges from 15,000 to 490,000, with an average of 114,000. Comparable results obtained with 9 immunized mice are listed in the last column of table 2. These values range from a minimum of 130 to a maximum of 2700, with an average of 1300 bacteria per gram of body weight. It is of interest to note that there is no significant change in the bacterial population in these immunized mice between 1 and 6 hours postinfection (compare last columns in tables 1 and 2), whereas the bacterial population in the control mice during the same time interval (columns 5, tables 1 and 2) shows a significant increase ($P>0.001$). Thus the induced active immunity is accompanied by an apparently stable bacterial population during this period and with the infectious dose employed.

A comparison of the relative increase

in numbers of bacteria between 1 and 6 hours postinfection in the different groups of mice shows the most rapid multiplication to have occurred in the animals given mucin as adjuvant. Using the average values shown for these mice in tables 1 and 2, calculation of the mean generation time for the in vivo multiplication of the bacteria renders a value between 23 and 24 minutes. This approximates the generation time observed for this organism grown in vitro at 37 C in brain-heart infusion broth (22 minutes). It is, of course, apparent that all bacteria destroyed by the body defenses of the mice during this 5-hour interval have failed to contribute their progeny to the final count. For this reason, the calculated generation time must be considered longer than the true value. Since the bacteria in all of the other experimental and control groups of mice fail to even approximate the rapid generation time found for "mucin" mice, it would appear that in some way mucin intereferes with the destruction of bacteria by body defenses, thereby leaving a greater growth potential of the pathogens. This interpretation is in line with the generally accepted explanation for the mode of action of mucin (see Olitzki, 1948).

3. *The number of* S. typhimurium, *strain V31, in the body of immunized mice 30 hours postinfection*

The number of bacteria per gram of body weight found 30 hours postinfection in 7 immunized mice infected with 50 million cells varied from a minimum of 176,000 to a maximum of 1,770,000; the average is 1 million. There is no significant difference between these results and those obtained from counts made 6 hours postinfection on immunized mice similarly infected (column 2, table 2). It can be seen from these data that in immunized mice the viable population reached early in the postinfection period, i.e., within 6 hours, remains unchanged for at least an additional 24 hours.

4. *The number of* S. typhimurium *in the body of the mice immediately after death*

Bacterial counts per gram of body weight determined immediately after death in 16 control mice infected with 50 to 80 million cells of *S. typhimurium*, strain V31, are given in column 1 of table 3, and are listed in order of increasing survival time, as shown by the parenthetic values in the same column. The average number of bacteria per gram of body weight was found to be 200 million, while the average survival time was 11 hours. Counts obtained with 14 immunized mice similarly infected are listed in column 1 of table 4. The average number of *S. typhimurium* per gram of body weight (35 million) is significantly lower than the number found in control mice ($P>0.001$). The average survival time (94 hours) was significantly larger ($P>0.001$).

Similar counts on 18 controls mice infected with 5 to 8 million cells of *S. typhimurium*, strain V31, are listed in column 2, table 3, and those for 13 mice infected with 200 thousand to 1 million cells are given in column 3, table 3. No significant difference was found in the pathogen population obtained with the mice infected with these dosages, even though the survival time is significantly longer ($P>.001$) for the animals infected with the smaller dose. The average counts per gram of body weight were 50 million and 42 million, whereas the average survival times were 70 hours and 128 hours. This suggests that survial time is related to the time necessary for the organisms to reach a relatively constant lethal population of viable

TABLE 3.—*The number of* S. typhimurium *found per gram of mouse at death in control mice following intraperitoneal infection with different dosages of the two strains. Survival time, in hours, is shown in parentheses with each individual value.*

Number of *S. typhimurium* ($\times 10^6$) found per gram of mouse at death in individual control mice surviving for hours shown ( ) following infection with:					
Strain V31 in dose of:			Strain Texas in dose of:		
50 to 80 $\times 10^6$	5 to 8 $\times 10^6$	0.2 to 1 $\times 10^6$	10 $\times 10^6$	1 $\times 10^6$	0.4 to 0.2 $\times 10^6$
83* (9)	63 (18)	20 (77½)	100 (13)	13 (21)	20 (74)
245* (9)	70 (25)	79 (77½)	120 (15)	31 (49½)	35 (83)
180* (9½)	19* (54)	47 (93)	242 (15½)	61 (57)	15 (95)
590* (9½)	24* (54)	11 (94)	83 (16½)	32 (60)	46 (97½)
116 (10)	10 (57)	26 (98½)	175 (17)	54 (74)	23 (99½)
130* (10)	112 (60)	39 (119)	247 (17)	57 (75)	61 (149½)
189 (10)	22* (62)	23 (120½)	157 (17½)	24 (82½)	
180 (10½)	38 (66½)	48 (143½)	103 (24)	60 (98½)	
140 (11)	141 (69½)	67 (144)		85 (125)	
186 (11½)	30 (75½)	88 (167)		55 (132½)	
194 (11½)	27 (77½)	27 (168)		101 (145)	
220 (11½)	55 (78)	24 (170)		75 (237½)	
195 (12)	28 (79)	41 (190)		45 (250)	
137 (12½)	27* (80)				
225 (13)	44 (95½)				
158 (19)	90 (99)				
	63 (102)				
	40 (124)				
Average 200 (11)	50 (70)	42 (128)	153 (17)	53 (108)	34 (100)

* Male mice.

cells. Similar results were obtained when the dosage was held constant, and survival time was altered by injections of metabolic inhibitors and intermediates (Berry, 1955).

Death counts per gram of body weight were also made on 20 mice infected with 5 to 8 million cells of *S. typhimurium*, strain V31, suspended in mucin. These results are listed in order of increasing survival times, parenthetic values, shown in column 2, table 4. The average count is 185 million cells per gram of body weight; the average survival time was 11 hours. Even though this survival was much lower than that of control mice given the same infectious dose (compare column 2, table 3), the death counts per gram of body weight were considerably higher ($P > 0.001$). On the other hand, there is no significant difference between the number of bacteria per gram of mouse found in this group and in the control group infected with 50 to 80 million cells (column 1, table 3). The average survival time was 11 hours for both groups.

Death counts per gram of body weight, obtained with 8 control mice infected with 10 million cells of the Texas strain, are listed in column 4, table 3 in order of increasing survival times (parenthetic values). The average count is 153 million; the average survival time is 17 hours. Comparable results obtained with 13 immunized mice given the same infectious dose are listed in column 3, table 4. The average count is 33 million; the average survival time is 144 hours. Death counts per gram of body weight were also made on 13 control mice infected with 1 million bacteria. The results are listed in column 5, table 3. Also shown are the results with 6 mice infected with 40 to 200 thousand cells of the same strain, column 6, table 3. The average counts for these groups are 53 and 34 million, respectively, and the average survival times are 108 and 100 hours. It is of interest to note that the counts obtained at death in these mice are significantly lower than those obtained when a dose of 10 million cells was used ($P > 0.001$), but they do not differ significantly from one another nor do they differ significantly when they are compared with results obtained with immunized mice (column 3, table 4).

Additional determinations were carried out on mice infected with 1 million

TABLE 4.—*The number of* S. typhimurium *found per gram of mouse at death in different experimental groups subjected to the treatments indicated. Survival time, in hours, is shown in parentheses with each value.*

Number of *S. typhimurium* ($\times 10^6$) found per gram of mouse at death in individual mice surviving for hours shown ( ) following infection with:					
Strain V 31 in:			Strain Texas in:		
Immune mice given 50 to 80 $\times 10^6$ cells	Mucin as adjuvant with 5 to 8 $\times 10^6$ cells	Immune mice given 10 $\times 10^6$ cells	Mice given 0.25 $\times 10^9$ heat-killed cells at infection with 10^6 cells	Mice given 0.5 $\times 10^9$ heat-killed cells at infection with 10^6 cells	Mice exposed to altitude for 3 weeks prior to infection with 10^6 cells
14 (56)	54 (8)	7 (49½)	61 (18)	68 (17)	61 (24)
7 (60½)	63 (10)	65* (74)	64 (20)	32 (18)	21 (28)
18 (61)	63 (10)	45* (102)	112 (25)	19 (18)	43 (70)
20 (62½)	126 (10)	23* (125)	90 (27)	63 (18)	18 (74)
20 (76)	130 (10)	7 (142½)	38 (31)	24 (19)	16 (74)
39 (84)	131 (10)	42* (149)	19 (48)	91 (21)	37 (75)
30 (85)	210* (10)	19* (149½)	27 (48)	73 (48)	28 (75)
51 (87)	361* (10)	37 (149½)	13 (70)	109 (23)	14 (76)
36 (96½)	466* (10)	56* (150)	13 (94)	13 (23)	
85 (101½)	137 (10½)	43* (154)		19 (27)	
46 (129½)	73* (11)	31 (169)		9 (41)	
51 (133)	131 (11)	20 (173)		21 (97)	
25 (135)	141 (11)	33 (174)			
45 (150½)	340 (11)				
	341 (11)				
	131 (12)				
	70 (12½)				
	178 (12½)				
	203 (12½)				
	300 (12½)				
Average 35 (94)	185 (11)	33 (144)	49 (42)	45 (26)	30 (62)

* Male mice.

cells of strain Texas that were administered concurrently with either ¼ or ½ billion heat-killed cells. The results are given in columns 4 and 5 of table 4. The number of viable bacteria at death are not significantly different from each other nor do they differ from those of control mice similarly infected (49 and 45 million, respectively, against 53 million, column 5, table 3). The mean survival times of 42 hours and 26 hours are not significantly different by rank order test, but they are significantly lower than that of control mice as shown in column 5, table 3 ($P>0.01$ for ¼ billion heat-killed cells and $P>0.001$ for ½ billion heat-killed cells). These results suggest that, under the specific conditions of the experiment, the number of dead pathogens fails to alter significantly the number of viable pathogens recoverable at death of the mouse.

Similar results were obtained with mice exposed to simulated altitude for three weeks prior to infection with 1 million cells of strain Texas, as shown in column 6 of table 4. The mean number of bacteria per gram of mouse at death, 30 million, does not differ significantly from that of control mice while their average survival time is significantly less ($P>0.05$). This implies that the lethal population of pathogens is reached more quickly in these animals than in control mice.

DISCUSSION

These results establish that at the time of death of mice artificially infected with *S. typhimurium* via the intraperitoneal route, the number of viable pathogens within the carcass of the mouse is comparatively constant regardless of the infectious dosage, the survival time, or alterations in the defense mechanism of the host except under those conditions where survival time averages no more than 17 hours. In such cases, significantly more organisms are present, but only by an amount that would be reached in two or three bacterial generations. Thus, in control mice infected

with 5 to 8 million, 1 million, 200 thousand or 40 thousand cells of either strain V31 or Texas, no significant difference was found in viable organisms at death even though the values for individual mice range from 10 to 141 million bacteria per gram of body weight, and their survival times ranged from a minimum of 18 to a maximum of 250 hours.

In contrast, when control mice were infected with 50 to 80 million cells of V31 or 10 million cells of the Texas strain, the recoverable bacteria at death ranged from 82 to 590 million bacteria per gram of body weight. All animals died within 24 hours. This survival time is significantly shorter than in the previous group. There is no way to account for the greater number of viable bacteria in mice surviving for the shorter periods of time than in those surviving longer. As Olitzki (1948) points out, when a massive infectious dosage is given, it is difficult to ascertain whether death is due to the virulence and invasiveness of the injected pathogens, or merely to the action of endotoxins liberated in the body through disintegration of the bacteria. On the basis of the results obtained with mice administered heat-killed cells at the time of infection, it would appear that the endotoxins per se fail to contribute significantly to the number of bacteria recoverable at death within the period of survival of these animals. One cannot assume, therefore, that variations in the number of bacteria destroyed within the host by its defense mechanisms contributes, at least statistically, to the observed differences in viable pathogens at the time of death unless it is further assumed that the defenses of the host have a comparatively fixed capacity for destruction of pathogens. It would have to be argued that under all the observed experimental conditions in which the same average number of pathogens were found at death, the same number of pathogens had been destroyed. This argument would lead, therefore, to the conclusion that with massive infectious doses of bacteria or with mucin as an adjuvant, fewer organisms are destroyed and hence the viable count is higher. That time alone is the primary factor determining the number of bacteria destroyed by body defenses cannot be supported in fact, since it has already been shown that metabolic inhibitors and intermediates fail to alter the viable population of *S. typhimurium* in the bodies of mice surviving for as little as 4½ hours or as long as 212 hours.

Mice given a single immunizing injection of vaccine were protected against the artificial infections as evidenced by their increased survival time, when compared with control mice similarly infected. However, the number of viable pathogens they contained at death was similar to that of control mice infected with smaller numbers of cells and surviving a comparable period of time.

Bacterial counts obtained with mice infected with mucin as an adjuvant were higher than those of control mice infected with the same number of cells, but were equivalent to those obtained in other control mice infected with approximately ten times as many pathogens. Mucin is generally referred to as a "virulence-enhancing" agent (Burrows, 1954), and an increase in virulence is often thought to be the result of an increase in toxicity (Burrows, 1954). Therefore, in terms of this concept of virulence, infection with mucin should result in a smaller population of bacteria at death. In these experiments this has not been found; in fact, the opposite result was obtained. Experiments using mucin which have been performed by other investigators (Olitzki, 1948)

seem to indicate that the action of mucin is due to its coating of the bacterial cell. As a result of this action of mucin evidence for the following effects have been presented: (a) inhibition of phagocytosis (Tunnicliff, 1940); (b) inhibition of intraphagocytic digestion (McLeod, 1941; Nungester, 1936); (c) inhibition of antibody uptake —it has been shown that mucin does not act as a hapten (Koefer and Spink, 1938; Olitzki et al, 1947); (d) inhibition of the bactericidal action of serum (Keefer and Spink, 1938; McLeod, 1941) and peritoneal fluid (Oerskov, 1940; Forthergill et al, 1937; Olitzki and Koch, 1945). This coating effect is believed to be due to the chemical nature of mucin (Smith, 1951). These experiments permit no conclusion as to the mode of action of mucin.

All bacterial counts made 1 and 6 hours postinfection after small infectious dosages (5 to 8 million or 1 million) show approximately the same fraction (1/5) of viable bacteria remaining one hour postinfection. The same fraction of viable cells is found in control mice infected with 50 to 80 million bacteria. However, the number of viable cells found in these control mice is significantly larger than that found in immunized mice given the same infectious dosage. It seems that the increased capacity of immunized mice to reduce the number of viable bacteria only becomes apparent when the system is confronted with a sufficiently large infectious dosage.

The size of the difference in the number of viable cells between 1 and 6 hours postinfection gives some indication of the rate at which the pathogens are approaching a lethal population. Thus, when there is a large increase by 6 hours, survival time is short; and, conversely, a small or negligible increase is followed by a longer survival time.

Results obtained with immunized mice show that there is no increase in the number of viable cells between 6 and 30 hours postinfection. This suggests that counts made at still longer intervals postinfection might reveal whether the pathogens increase over a number of hours to reach the number found at death, or rather remain "static" until only a few hours before death.

The two strains of *S. typhimurium* employed in these studies have approximately equal virulence as judged by survival times of control mice infected with comparable numbers of cells. They also show no statistical difference in the number of viable cells recoverable at death of animals (see table 3). It would be of interest to make similar determinations on strains of this species with widely differing virulence as far as infectious dosages are concerned to see whether the number of bacteria at death are also different.

SUMMARY

1. The number of viable cells of each of two strains of *Salmonella typhimurium* (V31 and "Texas") in the body of mice infected intraperitoneally was determined 1 and 6 hours postinfection and immediately after death.

2. Counts obtained 1 hour postinfection with control and immunized mice and mice infected with mucin as an adjuvant, show no significant difference when the groups compared have been infected with 5 to 8 million cells of strain V31. On the other hand, when control and immunized mice are infected with 50 to 80 million cells of the same strain, a significantly lower number of viable cells is found in the immunized mice.

3. Counts made 6 hours postinfection show a significant increase in the number of viable cells, compared with 1 hour counts, in control mice and mice

given mucin as an adjuvant, when infected with 5 to 8 million cells of strain V31. The counts in mice infected with mucin as an adjuvant are significantly larger, however, than those in control mice. Immunized mice infected with 1 million cells of *S. typhimurium*, strain Texas, show no significant increase in the number of viable cells between 1 and 6 hours postinfection, whereas the control mice do show a large increase. Immunized mice infected with 50 to 80 million cells of *S. typhimurium*, strain V31, show a significant rise in the viable bacterial population between 1 and 6 hours postinfection. This rise is not as great as that found in control mice during the same period.

4. Results obtained with immunized mice infected with 50 to 80 million cells of *S. typhimurium*, strain V31, show that an apparently stable viable population is maintained between 6 and 30 hours postinfection.

5. A relatively constant viable population is found at death in all the infected mice. Survival time was short (less than 24 hours) in control mice infected with massive dosages (50 to 80 million cells of *S. typhimurium*, strain Texas) and in mice infected with mucin as an adjuvant (5 to 8 million cells of *S. typhimurium*, strain V31). Bacterial counts per gram of body weight were significantly higher in these groups than in control mice infected with smaller dosages (5 to 8 million cells of *S. typhimurium*, strain V31, and 1 million cells of *S. typhimurium*, strain Texas) and immunized mice infected with 50 to 80 million cells of *S. typhimurium*, strain V31, or 10 million cells of *S. typhimurium*, strain Texas. An inverse relationship was found between survival times of about 12 hours versus more than 24 hours and the size of the viable bacterial population at death, even though the difference in number of viable bacteria represented only two to three cell generations.

6. The viable population of strain Texas at death of mice given either $\frac{1}{4}$ billion or $\frac{1}{2}$ billion heat-killed cells at the time of infection is not significantly different from that of control mice even though the survival time of these animals is significantly less. Similar results were obtained with mice exposed to simulated altitude for three weeks prior to infection.

REFERENCES

Berry, L. J. and Mitchell, R. B. 1953a, Tex. Rep. Biol. & Med. **11**: 379–401.

———. 1953b, J. Infect. Dis. **93**: 75–82.

———. 1953c, J. Infect. Dis. **93**: 83–92.

Berry, L. J., Merrit, P. and Mitchell, R. B. 1954, J. Infect. Dis. **94**: 144–151.

Berry, L. J. 1955, Ann. N. Y. Acad. Sc. In press.

Burrows, W. 1954, A Textbook of Bacteriology, Philadelphia, W. B. Saunders Co.

Fothergill, L. D., Dingle, J. H. and Chandler, C. A. 1937, J. Exper. Med. **65**: 721–734.

Keefer, C. S. and Spink, W. W. 1938, J. Clin. Investigation, **17**: 23–30.

McLeod, C. 1941, Am. J. Hyg., sec. B, **34**: 51–63.

Nungester, W. J., Jourdonais, L. F. and Wolf, A. A. 1936, J. Infect. Dis. **59**: 11–21.

Oerskov, J. 1940, Ztschr. f. Immunitätsforsch. **98**: 359–372.

Olitzki, L. and Koch, P. K. 1945, J. Immunol. **50**: 229–235.

Olitzki, L., Shelubsky, M. and Efrati, E. 1947, Proc. Soc. Exper. Biol. & Med. **64**: 258–259.

Olitzki, L. 1947, Bact. Rev. **12**: 149–172.

Smith, H. 1951, J. Infect. Dis. **88**: 207–214.

Tunnicliff, R. 1940, J. Infect. Dis. **66**: 189–191.

White, C. 1952, Biometrics, **8**: 33–41.

STUDIES ON THE BIOLOGY OF *CRYPTOCOCCUS*

VI. AMINO ACID COMPOSITION OF THE SOMATIC PROTEINS OF *CRYPTOCOCCUS NEOFORMANS*

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The antigenic properties[1,2] and the polymeric nature[3] of capsular polysaccharides of *Cryptococcus neoformans* have been established. Little is known, however, of the somatic proteins of these organisms. The present study was undertaken to establish what amino acids *C. neoformans* can synthesize and incorporate into its somatic proteins from an inorganic source of nitrogen, and to ascertain if peculiarities in the composition of these proteins may provide clues for further exploration of the biosynthetic pathways involved, or serve as a guide in distinguishing the pathogenicity of different strains.

METHODS

Two strains of *C. neoformans* previously isolated from cases of human cryptococcosis were used. Both were in the M phase[4] but differed in their degree of encapsulation on Sabouraud's agar. Studies on the capsular polysaccharides of these two strains have been recorded previously.[3]

These strains were grown in ammonium sulfate-glucose broth (with thiamine hydrochloride, 0.2 μg/ml) as used by Mager[4,5] and Hehre et al[6] at 25 C with constant aeration for 10 days. The cells were harvested and decapsulated in the manner previously described,[3] except that 4*N* sodium hydroxide was used in the decapsulation process. The precipitate ensuing after neutralization with 6*N* hydrochloric acid was combined with that resulting from the addition of trichloracetic acid. The combined precipitates were washed with 70% ethanol several times and dried in vacuo to yield a white, very brittle powder. This material was quite insoluble in water and salt solutions.

Twenty mg of this cell-protein powder from the heavily encapsulated strain (C-94) and the lighter encapsulated strain (C-JM) were hydrolyzed in sealed tubes with 1.0 ml of 6*N* hydrochloric acid and 0.5 ml glacial acetic acid for 24 hours at 110 C. The hydrolysates were taken to dryness in vacuo repeatedly to remove the acid, and the final residues were dissolved in 10 ml of water, filtered to remove the humin, and aliquots were used for paper partition chromatography, nitrogen analysis, and quantitative ion exchange chromatography of amino acids, according to the methods described by Moore and Stein.[7] The resin used was 8% cross-linked Dowex-50. The

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1. Neill, J. M., Castillo, C. G., Smith, R. H. and Kapros, C. E. 1949, Capsular reactions and soluble antigens of *Torula histolytica* and of *Sporotrichum schenckii*. J. Exper. Med. **89**: 93–106.
2. Neill, J. M., Abrahams, I. and Kapros, C. E. 1950, A comparison of the immunogenicity of weakly encapsulated and of strongly encapsulated strains of *Cryptococcus neoformans* (*Torula histolytica*). J. Bact. **59**: 263–275.
3. Foley, G. E. and Uzman, L. L. 1952, Studies on the biology of cryptococcus. IV. Isolation of a highly polymerized polysaccharide from encapsulated strains. J. Infect. Dis. **90**: 38–43.
4. Mager, J. and Aschner, M. 1947, Biological studies on capsulated yeast. J. Bact. **53**: 283–295.
5. Mager, J. 1947, Studies on the polysaccharides of capsulated yeasts. Biochem. J. **41**: 603–609.
6. Hehre, E. J., Carlson, A. S. and Hamilton, D. M. 1949, Crystalline amylose from cultures of a pathogenic yeast (*Torula histolytica*). J. Biol. Chem. **177**: 289–293.
7. Moore, S. and Stein, W. H. 1951, Chromatography of amino acids on sulfonated polystyrene resins. J. Biol. Chem. **192**: 663–681.

quantitation of eluates was effected using a modification of the colorimetric method of Yemm and Cocking.[8] The identities of the amino acids eluted from the columns were checked by paper chromatography. The absence of methionine in the hydrolysate was confirmed by the use of circular paper chromatography, using butanol-glacial acetic acid-ethanol-water (45:10:35:10) as the developing solvent. In this system methionine gives a distinct, well-resolved band, the absence of which can be unequivocally ascertained by comparison with control systems. When it became evident that methionine was absent in the hydrolysates of these cell-protein preparations, a second hydrolysis of the proteins was undertaken under identical conditions, except that 1.0 mg of methionine was added to each sample prior to hydrolysis. Paper chromatographic analysis of these hydrolysates showed the presence of methionine in the expected amount (semiquantitatively), indicating that it is unlikely that methionine was destroyed during the course of hydrolysis.

RESULTS

The amino acid composition of the somatic proteins of the two strains of *C. neoformans* studied is presented in table 1. It is evident that although the two strains differ in degree of encapsulation, their cell-body proteins show similar amino acid composition. The striking features are the high content of the dicarboxylic amino acids (aspartic and glutamic acids), and the absence of methionine. Although the cystine eluted from the chromatographic columns was too low to be measured, its presence was manifest on paper chromatograms. It must be assumed, therefore, that cystine occurs in trace amounts; thus sulfur-containing amino acids are not entirely absent from the somatic proteins of *C. neoformans*.

DISCUSSION

The similarity in the amino acid composition of the somatic proteins of the two strains studied was not surprising, since strain differences would have to be sought a priori in more subtle compositional differences than those investigated. The absence of methionine and the low sulfur content of the cell bodies do raise the question, however, as to whether the sulfur requirement plays a crucial role in the metabolism of these organisms. The high content in dicarboxylic amino acids suggest a highly acidic composition of the body proteins of *C. neoformans*. Even if part of the dicarboxylic amino acid residues were present as glutamine and asparagine in the protein structure, as would appear from the ammonia liberated by hydrolysis (table 1), the acid nature of the total proteins would still be maintained unless one envisaged extensive amide cross-linking between peptide chains of the glutaminyl-aspartyl, asparaginyl-glutamyl type. The insolubility of the body-proteins of *C. neoformans* (as prepared here), may be a manifestation of such extensive cross-linking in the protein structure of the organism.

TABLE 1.—*Amino acid composition of two strains of* C. neoformans.

Amino acid	Strain C-JM (light capsule)		Strain C-94 (heavy capsule)	
	Micromoles per 10 ml hydrolysate	Percent of total*	Micromoles per 10 ml hydrolysate	Percent of total*
Aspartic acid	2.60	10.0	2.58	9.3
Threonine	1.66	5.7	1.65	5.5
Serine	2.06	6.0	1.81	5.2
Glutamic acid	2.60	11.0	2.72	10.9
Proline	1.58	5.2	1.15	3.6
Glycine	2.02	4.3	2.08	4.3
Alanine	2.44	6.3	2.64	6.3
Valine	1.90	6.3	2.08	6.5
Cystine	<0.10†		<0.10†	
Methionine	0†	0	0†	0
Isoleucine	1.75	6.7	2.24	7.9
Leucine	2.98	11.2	3.16	11.2
Tyrosine	0.39	2.0	0.49	2.5
Phenylalanine	1.01	4.9	1.26	5.7
Histidine	0.42	2.0	0.61	2.5
Lysine	1.86	7.8	2.00	7.9
Arginine	1.20	6.0	1.07	5.2
Ammonia	8.65‡	4.3	11.05‡	5.2

* By weight of amino acid.
† Both cystine and methionine were undetectable by ion exchange chromatography. A trace of cystine was found by paper chromatography.
‡ A portion of the ammonia may have arisen from tryptophane, destroyed in the acid hydrolysis.

8. Yemm, E. W. and Cocking, E. C. 1955, The determination of amino acids by ninhydrin. Analyst, **80**: 209–213.

SUMMARY

The somatic total proteins of a heavily encapsulated strain and a lightly encapsulated strain of *Cryptococcus neoformans* were isolated. The amino acid composition of each was determined by quantitative ion exchange chromatography. The over-all composition of the two strains proved similar. Both strains were distinguished by a high content of dicarboxylic amino acids and the absence of methionine.

HEMOLYSIS AND HEMAGGLUTINATION BY NORMAL AND IMMUNE SERUMS OF ERYTHROCYTES TREATED WITH A NONSPECIES SPECIFIC BACTERIAL SUBSTANCE

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Some time ago a program was instituted to determine whether antibodies reacting with various streptococcal fractions and products could be detected in human serums by the method of hemagglutination or its hemolytic modification. Erythrocytes of various species were exposed to the materials derived from streptococci and the tests carried out by a modification of the methods of Middlebrook.[1]

In the course of this investigation a substance was discovered in broth culture filtrates of group A hemolytic streptococci which prepared erythrocytes for hemolysis or agglutination in the presence of certain human serums. Subsequent study revealed that a similar material was present in filtrates obtained from other gram positive, but not gram negative, organisms. A preliminary report of these observations has been made.[2] This paper describes in greater detail the properties of this nonspecies specific substance which is hereafter called "NSS."

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1. Middlebrook, G. 1950, A hemolytic modification of the hemagglutination test for antibodies against tubercle bacillus antigens. J. Clin. Investigation, **29**: 1480–1485.

2. Rantz, L. A., Zuckerman, A. and Randall, E. 1952, Hemolysis of red blood cells treated by bacterial filtrates in the presence of serum and complement. J. Lab. & Clin. Med. **39**: 443–448.

MATERIALS AND METHODS

Erythrocytes.—Sterile defibrinated sheep blood was obtained from a commercial source weekly, stored in the refrigerator, and discarded after 5 days. Human and rabbit blood were obtained as needed being drawn in modified Alsever's solution and used within a few days. All cells were washed three times with buffered saline immediately before use.

Human serums.—The human serums used throughout this investigation were obtained aseptically from healthy medical students and stored at −20 C in the deep freeze until used.

Buffered saline.—Nearly all washing of erythrocytes and dilutions of serum were made using an isotonic buffered saline solution at pH 7.0 and of the following composition per liter: Na_2HPO_4, 5.7 g, KH_2PO_4, 3.63 g, NaCl, 4.53 g.

Broth.—Liver digest broth was prepared by the digestion of chopped hog livers by chopped hog stomachs at 50 C at a pH of 2.0 overnight. Neutralization and clarification were carried out just before bottling and terminal sterilization in the autoclave. The final pH was circa 7.2.

Tryptose broth consisted of 20.0 g tryptose (Difco), 1.0 g beef extract, 1.0 g yeast extract, 2.0 g dextrose, and 5.0 g sodium chloride, and 2.5 g Na_2HPO_4 dissolved in 1 liter of water. The pH was adjusted to 7.2 before autoclaving.

Culture "filtrates."—Various organisms were grown in liver digest broth, usually for 4 days, the organisms were removed by centrifugation for 30 minutes at 3000 r.p.m., and the pH adjusted to 7.0 with 1*N* NaOH using a glass electrode. Subsequently in certain instances the material was sterilized by passage through a Seitz filter. In either instance the product was stored in 10 ml amounts at −20 C.

Acid extracts.—Extracts were prepared of various organisms that had been grown in tryptose broth for 24 hours and recovered by centrifugation. Ten ml of the extracting solution were used per liter of the original culture and were added to the sediment which was suspended by shaking and placed in a boiling water bath for 10 minutes. The process was repeated three times and the extracts were pooled and stored at −20 C.

The extracting solution in nearly all instances consisted of 2/*M* HCl in normal saline. In some experiments isotonic phosphate buffers for appropriate pH were utilized.

Phenol extracts.—The group A, type 12 streptococcus K 60 E was grown in 30 liters of tryptose broth. The organisms were removed in the Sharples' centrifuge, dehydrated in 200 ml of 95% ethyl alcohol, and soaked in 90 ml of 88% phenol for 24 hours at 37 C. The cell bodies were removed and re-extracted for a similar period of time with an additional 45 ml of phenol. The two extracts were pooled.

The extracts were treated successively with 10%, 1.0 and 2.0 volumes of ethyl alcohol in the cold. Precipitates had formed within 18 hours in each instance. These were removed in the centrifuge, dissolved in 88% phenol, reprecipitated with alcohol in the presence of a little sodium acetate, and the precipitates dried in a desiccator. An attempt was made to dissolve each in normal saline in a volume equal to about 5% of the original extract. Much of the material failed to dissolve and was removed in the centrifuge.

Immunization of rabbits.—A strain of group A hemolytic streptococcus (Banks), of *Micrococcus pyogenes* var. *aureus* (XX 152), and of *Escherichia coli* (40069) were each grown in 500 ml of tryptose broth, and the organisms were recovered by centrifugation and washed two times with a large volume of normal salt solution.

The organisms were finally suspended in about 5 ml of normal saline and heated at 65 C for 100 minutes. All of the vaccines were then sterile. Phenol to a final concentration of 0.25% was added as a preservative.

Serum was obtained from three normal rabbits and four weekly intravenous injections of 0.5 ml of the above vaccines were made. One week later another bleeding was done and the immune serums were obtained and stored at −20 C.

Techniques of the test.—The demonstration of hemolysis of treated erythrocytes required serum containing antibody and complement. The procedure was essentially that described by Middlebrook.[1]

Human and animal serums.—Serums were initially diluted appropriately with buffered isotonic salt solution, usually either 1:4 or 1:10. Each was then absorbed for 10 minutes at room temperature with 5.0% by volume of the species of red cell to be used in the test to remove heterologous antibodies. No adsorption was necessary when human group O cells and human serums were utilized. The adsorbing cells were removed by centrifugation.

Complement.—The test required a potent complement. Fresh pooled guinea pig serum was the most satisfactory. It was received on the day of collection, stored at −20 C, and discarded after 5 days. Before use it was diluted 1:3 with buffered isotonic salt solution and absorbed twice at 4 C for 10 minutes with 5.0% by volume of erythrocytes of the same species as were to be used in the test. The cells were removed by centrifugation at 4 C. Complement was adsorbed with red cells sensitized with solutions rich in the C carbohydrate of group A hemolytic streptococci when experiments using erythrocytes exposed to acid extracts were carried out. The formamide extraction method was used to prepare the polysaccharide.

Many failures occurred early in the study when the complement was not processed throughout in the cold room. The rise in temperature during centrifugation at room temperature resulted in substantial loss of activity.

Sensitization of erythrocytes.—The bacterial filtrates and extracts were appropriately diluted with buffered salt solution and were heated at 56 C for 15 minutes to destroy thermolabile hemolysins. Thrice washed erythrocytes of the species being studied were suspended therein in a concentration of 2.0% by volume and incubated in a water bath at 37 C for 30 minutes to 2 hours. The time required for sensitization was studied and the results will be described later in this report.

After incubation the cells were washed three times with buffered isotonic salt solution and suspended in the same diluent to a final concentration of 0.5%.

The hemolytic test.—The serums were diluted serially with buffered isotonic salt solution and 0.5 ml of each dilution was pipetted into a 10 mm ×100 mm test tube to which was added 0.5 ml of the suspension of sensitized erythrocytes. One drop of absorbed complement was added to each tube and the whole was incubated in a water bath at 37 C, with frequent shaking for 1 hour. The tubes were centrifuged and the presence or absence of hemolysis was noted. End points were recorded as the last tube showing definite hemolysis. Titers were recorded as the reciprocal of the initial dilution of serum before adding the erythrocyte suspension.

The agglutination test.—Agglutination of the sensitized erythrocytes was demonstrated by a method exactly like that described above except that no complement was added. Tubes containing the serum dilutions and erythrocytes were well shaken and then allowed to stand undisturbed at room temperature for two hours. The

presence of agglutination was determined by application of the "pattern" technique.[3]

GENERAL PROPERTIES OF THE NON-SPECIES SPECIFIC SUBSTANCE

The basic observations which led to the discovery of NSS in culture filtrates which prepared erythrocytes for hemolysis in the presence of human serum and complement have been described.[2]

Two strains of group A hemolytic streptococci freshly isolated from the respiratory tracts of infected human beings were used for the preparation of filtrates which were used for the sensitization of erythrocytes. Hemolysis occurred in the presence of certain human serums. A typical experiment is depicted in table 1.

Agglutination of the sensitized erythrocytes occurred in the absence of complement and was studied in many of the experiments to be described. Generally the results were similar. Most of the work was done using the hemolytic system because it was more simple, particularly with reference to the end point of the reaction in systems including serum dilutions.

It was originally supposed that the active substance was specific for the streptococcus but this was soon discovered not to be the case. Filtrates prepared from most gram positive bacteria but no gram negative ones were similiarly active. The details of the experiments which support these statements will be presented shortly. At this time certain properties of NSS will be described as determined by study of filtrates and extracts derived from the Banks strain of group A streptococcus.

Production and stability of MSS in

TABLE 1.—*Hemolysis of sheep red cells treated by exposure to two different group A hemolytic streptococcal filtrates in the presence of human serum and complement.*

Serum	Untreated Serum Dilution	Hemolysis of sheep cells treated with: Banks filtrate Serum dilution									Espinosa filtrate Serum dilution								
		4	8	16	32	64	128	256	512	1024	4	8	16	32	64	128	256	512	1024
MS 32	0	+	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0
Zuck	0	+	+	+	0	0	0	0	0	0	+	+	+	0	0	0	0	0	0
MS 11	0	+	+	+	+	+	0	0	+	0	+	+	+	+	0	+	0	+	+
Bodds	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+++, maximum hemolysis.

3. Denny, F. W., Jr. and Thomas, L. 1953, The demonstration of type specific streptococcal antibody by a hemagglutination technique employing tannic acid. J. Clin. Investigation, **32**: 1085–1093.

filtrates.—NSS appeared in large amounts after 24 hours of incubation of the culture, persisted at the same level for an additional three days and then declined, none being demonstrable after 9 days at 37 C. Production was comparable in liver digest and tryptose broths.

In other studies, some of which were conducted with acid extracts of the streptococcus and some with filtrates, it was demonstrated that a critical amount of NSS was required for sensitization. Thus 10 ml of a 1:4 dilution of a particular extract were fully effective in the sensitization of 0.2 ml of packed sheep erythrocytes. There was a progressive loss of activity when the volume was reduced to 6.0 and 2.0 ml. No hemolysis occurred when only 1.0 ml was employed.

It was also demonstrated that the ability of filtrates and extracts to prepare erythrocytes for participation in the reaction was exhausted by exposure of these cells. Ten ml of a 1:2 dilution of streptococcus filtrate or a 1:8 dilution of the extract described above were incubated with 2.0 ml of packed sheep erythrocytes for 2 hours, following which the cells were removed by centrifugation. These adsorbed materials were then used to sensitize 0.2 ml of fresh sheep red cells and were found to be almost without activity in the hemolytic system. The concentration of M protein and C carbohydrate of the group A streptococcus estimated by antigen dilution precipitin tests using serums obtained from the U. S. Public Health Service Communicable Disease Center, Chamblee, Georgia, was not diminished by exposure to this amount of erythrocytes.

The active substance was quite heat stable at acid pH. It withstood 100 C for 15 minutes at pH 2.0, 4.0, and 6.0, but was completely inactivated at pH 7.0 and 8.0. At neutrality it could be exposed to 56 C for 3 hours and 80 C for 30 minutes without loss of potency. The active principle was not removed by passage of the culture through Zeitz or sintered glass filters.

Human, horse, rabbit, and sheep erythrocytes were employed in the test. Only the latter were hemolyzed in the presence of complement but all four were comparable when agglutination was studied. Initially the erythrocytes were exposed to the filtrates for 2 hours during sensitization but it was subsequently demonstrated that 5 minutes was equally effective. A 30 minute interval was employed during much of the latter work.

Presence of NSS in extracts of bacteria.—Nearly all of the observations just described were made using a culture filtrate of a strain of group A streptococcus. During experiments designed to sensitize erythrocyctes with specific streptococcal substances, it was discovered that acid extracts of these organisms of the familiar Lancefield type used for serological grouping and typing were also rich in NSS.

It was discovered that most pools of guinea pig serum used as a source of complement contained a substance, presumably an antibody, which caused hemolysis of erythrocytes exposed to acid extracts and to solutions of C carbohydrate prepared by the formamide method. Such complement caused hemolysis in all tubes when used in the test. This could be obviated by adsorption of the guinea pig serum in the cold with sheep cells sensitized with C carbohydrate. This phenomenon was studied in detail and will be the subject of another communication.

A typical experiment demonstrating NSS in acid extracts is presented in table 2. The group A, type 12 streptococcus, K 60 E, was extracted at circa

TABLE 2.—*Hemolysis of sheep erythrocytes sensitized by a 1:2 dilution of a culture filtrate and a 1:8 dilution of a pH 2.0 extract of the group A streptococcus "Banks."*

Human serum	Hemolysis of sheep cells treated with: Filtrate Serum dilution						Acid extract Serum dilution						Untreated Erythrocytes
	4	8	16	32	64	128	4	8	16	32	64	128	
MS 184	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	++	0
MS 200	+++	+++	+++	+++	+++	0	+++	+++	+++	+++	+++	+	0
MS 180	0	0	0	0	0	0	0	0	0	0	0	0	0
MS 191	+++	0	0	0	0	0	+++	+	0	0	0	0	0
MS 195	0	0	0	0	0	0	0	0	0	0	0	0	0

+++, maximum hemolysis.

pH 2.0 at 100 C, and this material was compared with a culture filtrate derived from the same organism using a "battery" of five human serums in the hemolytic test. Serums MS 184 and 200 contained large amounts, and serums MS 180, 191, and 195 little or none of the antibody which is operative in this system. The similarity of the titers with both sets of treated red cells is evident.

Experiments designed to determine the optimum pH of extraction demonstrated maximal amounts in those prepared at pH 1.7 and 3.8. A rapid decline in potency with decrease in acidity was doubtless due to the instability of NSS to heat in neutral and alkaline solutions.

Extracts could be diluted 1:8 without loss of activity and were capable of producing some sensitization of erythrocytes in a dilution of 1:32.

A simple fractionation of an acid extract of "Banks" was attempted by the method of Lancefield.[4] A precipitate was formed by the addition to the extract of 20 g per liter of sodium acetate and 4 volumes of 95% ethyl alcohol. When this had been redissolved in about 10% of the original volume of extract and the insoluble residue removed in the centrifuge, it contained a large amount of M protein but no NSS. The volume of the supernatant was reduced to 10% of the original volume of extract by evaporation, and the large amount of sodium acetate and other salts were removed by dialysis in the cold in a cellophane bag. The resulting solution was made isotonic by the addition of an appropriate amount of sodium chloride. This fraction now contained very large amounts of the C carbohydrate but no NSS.

These observations suggested that the NSS might well be dialyzable but this was subsequently demonstrated not to be so by other experiments using untreated extracts and filtrates. The concentration of NSS was not reduced after 24 hours of exposure of the materials to cold distilled water in cellophane sausage casings.

Further attempts at fractionation of acid extracts were not undertaken and a satisfactory explanation of the loss of NSS during the procedures described above was not obtained.

Other experiments demonstrated that the NSS in filtrates and acid extracts had comparable heat stability and was capable of sensitizing the same species of erythrocytes for the hemolytic reaction.

Phenol extracts.—A type 12 hemolytic streptococcus was extracted in phenol. Material for study was recovered by precipitation with 0.1, 1.0, and 2.0 volumes of ethyl alcohol and was dissolved in normal saline.

Results of immunologic study of these three fractions are presented in

4. Lancefield, R. C., 1928, The antigenic complex of *Streptococcus haemolyticus*. I. Demonstration of a type specific substance in extracts of *Streptococcus haemolyticus*. J. Exper. Med. **47**: 91–103.

table 3. Practically all of the two specific streptococcal substances—the C carbohydrate and M protein—were contained in the 1.0 volume fraction. Even here the amounts, as estimated by antigen dilution tests with specific serum, were not great when the large amount of culture extracted was considered. Much greater concentration of both were present in the acid extracts previously described.

All three fractions contained NSS. The two volume fraction seemed to be the most potent and was fully effective in a dilution of 1:16. The original volume of precipitate in this fraction as estimated by inspection had been quite small, and it seemed evident that the greatest purification and concentration of NSS had occurred here.

The fact that NSS was readily recovered by solution of the streptococcus in phenol and precipitation with alcohol and acetate suggested that it might well be a polysaccharide. Trypsin digestion of the pooled 1.0 and 2.0 volume fractions were undertaken. Two 1.0 ml portions of the extract were diluted 1:5 with a 0.2% solution of crystalline trypsin (Nutritional Biochemicals Co.) in buffer at pH 7.2. The trypsin used for dilution of one portion had been inactivated at 60 C for 15 minutes. Both were incubated at 37 C for 3 hours and were tested for NSS content. No loss of activity as the result of exposure to active trypsin was demonstrated.

All three of the alcohol precipitates of the phenol extract also contained another quite different substance capable of sensitizing erythrocytes for a hemagglutination reaction with human serums. It will be described elsewhere.

Presence of NSS in filtrates and extracts of organisms of various species.—Soon after the discovery of NSS it was noted that it was not present solely in filtrates and extracts of group A strepto-

TABLE 3.—*Content of NSS and specific streptococcal antigens in alcohol fractions of phenol extract of a group A, type 12, hemolytic streptococcus.*

Alcohol fraction	Hemolysis of treated erythrocytes in presence of human serum													Precipitation in presence of specific streptococcal serums								
	MS 190—serum dilution					Ande—serum dilution								Group A "C"					Type 12 "M"			
														Antigen dilution					Antigen dilution			
	4	8	16	32	64	128	4	8	16	32	64	128	UD	2	4	8	16	UD	2	4
0.1 volume	+++	+++	+++	+++	0	0	+++	+++	+++	0	0	0	0	0	0	0	0	0	0	0
1.0 volume	+++	+++	+++	+++	0	0	+++	+++	+++	0	0	0	+	++	+	+	0	+	+	0
2.0 volume	+++	+++	+++	+++	++	+++	+++	+++	+++	++	0	0	+	0	0	0	0	0	0	0

+++, maximum hemolysis or precipitation.
UD, undiluted.

cocci but could also be obtained from many gram positive but no gram negative bacteria. This matter was studied systematically first with culture filtrates of a number of organisms and later using hot $M/20$ HCl extracts of a large number of strains of several species of bacteria.

These extracts or filtrates were used for the sensitization of sheep erythrocytes which were employed in the hemolytic test with batteries of nine human serums. (The battery used in testing the filtrates was comparable in structure but composed of different serums.) These were so selected that four had very high, and five very low, titers of the antibody which reacted in the system with cells treated with the Banks filtrates and K 30 E extracts used in the experiments described above.

Table 4 depicts the type of data obtained. Information presented was obtained from extracts, but filtrates gave exactly comparable results, as is demonstrated in line 1 of the table. It will be observed that serums 181, 184, 190, and 204 in high dilution uniformly hemolyzed erythrocytes sensitized with extracts of hemolytic and nonhemolytic streptococci, micrococci and bacilli. Serum 191 was in an intermediate position, and serums 180, 206, 193, and 194 were either inert in a 1:4 dilution or were active only in high concentration (line 1 through 6, table 4). A strain of enterococcus presented the same general pattern but its sensitizing effects were feeble. Pneumococci were difficult to study because hemolysis of the erythrocytes frequently occurred during sensitization. The example presented suggests the pattern of the first six organisms but is not identical.

The last seven organisms described are gram negative bacilli. These organisms were either incapable of sensitizing the erythrocytes (lines 11, 14, 15, and 17), or did so feebly and with a reaction pattern quite different from that of the gram positive organisms described above (lines 12, 13, and 16).

Using this technique, the filtrates of 36 and the extracts of 16 different organisms were tested. The results are summarized in table 5. The pattern of reaction with the serum batteries was readily segregated into four categories. "Typical" results presented in table 5 were similar to those obtained in lines 1 through 7 of table 4, "weak typical" results, like those in line 9, "inert" like those in lines 8, 11, 12, 14, 15, and 17, and "irregular," like those represented in lines 10, 12, 13, and 16 of the same table. Technical failures occurred with

TABLE 4.—*Hemolysis of sheep erythrocytes treated with extracts of various bacterial species by a battery of human serums.*

Cells treated with extract:	Maximum dilution of serum permitting hemolysis								
	Serum no.								
	181	184	190	204	191	180	206	193	194
1. "Banks" (standard filtrate)	64	64	128	64	4	<4	<4	<4	<4
2. Nonhemolytic streptococcus no. 86452	64	64	128	64	<4	<4	<4	<4	<4
3. Nonhemolytic streptococcus no. 86460	32	32	64	32	<4	<4	<4	<4	<4
4. *M. pyogenes* var. *aureus*, no. 88407	128	128	256	128	8	<4	<4	4	4
5. *M. pyogenes* var. *aureus*, no. XX392	128	128	128	128	16	<4	4	8	4
6. *M. pyogenes* var. *aureus*, no. XX396	128	128	256	128	16	<4	<4	4	4
7. *B. subtilis*, no. 88638	64	64	128	64	8	<4	<4	<4	<4
8. *Cl. welchii*	<4	<4	<4	<4	<4	<4	<4	<4	<4
9. Enterococcus, no. 85193	8	8	8	8	0	0	0	0	0
10. Pneumococcus	8	64	32	16	4	4	4	4	4
11. *Esch. coli*, no. 88193	<4	<4	<4	<4	<4	<4	<4	<4	<4
12. *Esch. coli*, no. 88443	<4	<4	<4	4	<4	4	4	8	4
13. *Esch. coli*, no. 40815	<3	4	<4	16	<4	4	<4	<4	<4
14. Pseudomonas sp., no. 88375	<4	<4	<4	<4	<4	<4	<4	<4	<4
15. Pseudomonas LB 2	<4	<4	<4	<4	<4	<4	<4	<4	<4
16. Proteus, no. 88729	4	4	<4	4	4	4	4	4	4
17. Salmonella sp., no. 88642	<4	<4	<4	<4	<4	<4	<4	<4	<4

TABLE 5.—*Presence of NSS in filtrates or extracts from 85 strains of various species of microorganisms.*

Organism	Number of strains	Sensitizing material	Serum antibody pattern				
			Typical*	Weakly typical*	Inert*	Irregular*	Technical failure
Hemolytic streptococcus, group A	12	Filtrate	12				
Hemolytic streptococcus, group A	2	Extract	2				
Hemolytic streptococcus, group C	1	Extract	1				
Hemolytic streptococcus, group G	1	Extract	1				
Str. viridans	1	Filtrate	1				
Str. viridans	3	Extract	3				
Enterococcus	3	Filtrate		3			
Enterococcus	2	Extract		2			
M. pyogenes var. *aureus*	4	Filtrate	4				
M. pyogenes var. *aureus*	13	Extract	12				
Pneumococcus	1	Filtrate	1				
Pneumococcus	6	Extract				1	5
Bacillus sp.	1	Filtrate	1				
Bacillus sp.	1	Extract	1				
Clostridium sp.	1	Extract			1		
Esch. coli	6	Filtrate			3	3	
Esch. coli	4	Extract				1	3
Proteus sp.	2	Filtrate			2		
Proteus sp.	3	Extract			1	2	
Pseudomonas sp.	5	Filtrate			4		
Pseudomonas sp.	7	Extract			7		
Salmonella sp.	1	Filtrate			1		
Gonococcus	1	Extract			1		

* For explanation of term see text.

extracts, but not filtrates, of five strains of pneumococcus and three of *Esch. coli* because the extracts were hemolytic for erythrocytes.

All strains of groups A, C, and G streptococci, of *Streptococcus viridans*, of *M. pyogenes* var. *aureus*, and bacillus sp. produced extracts and filtrates that sensitized erythrocytes to react with serums in exactly the same way as did the type organism with which the basic observations had been made. Similar results were obtained with the filtrate of one pneumococcus. The extract of another gave the pattern depicted in line 10 of table 4 which resembles but is not identical with the "typical" one. Enterococcus behaved similarly but the serum titers were always less when tested with erythrocytes exposed to filtrates or extracts derived from these organisms (line 9, table 4).

Filtrates and extracts of all pseudomonas sp., and of single strains of salmonella sp., gonococcus, and clostridium sp. were inert. Proteus and *Esch. coli* were either inert or gave irregular results. The latter indicated the presence of other antibodies, perhaps specific for *Esch. coli* in certain serums as has been described by Neter.[5,6]

Immunization of animals.—Later the evidence will be presented which indicates that the substance in certain human serums which hemolyzes the treated erythrocytes in the presence of complement is an antibody. It was desirable to learn whether injection of organisms in an experimental animal would simulate its production.

Rabbits were injected with a heat-killed vaccine prepared from strains of group A streptococcus, *M. pyogenes* var. *aureus*, and *Esch. coli*. These were then tested against heterologous and homologous group A streptococcal filtrates and extracts obtained from *M. pyogenes* var. *aureus*, and *Esch. coli*. The results of a typical experiment are depicted in table 6. The serums used are

5. Neter, E., Bertram, L. F., Zak, D. A., Murdock, M. R. and Arbesman, C. E. 1952, Studies on hemagglutination and hemolysis by *Escherichia coli* antisera. J. Exper. Med. **96**: 1–15.

6. Neter, E., Zalewski, N. J. and Ferguson W. W. 1953, *Escherichia coli* hemagglutinin response of adult volunteers to ingested *E. coli*. Proc. Exper. Biol. & Med. **82**: 215–219.

those obtained after immunization. None drawn before this procedure contained the hemolysin.

The data demonstrate clearly that serums from rabbits immunized with streptococci and micrococci react with erythrocytes sensitized by exposure to extracts or filtrates of these organisms. No evidence of strain specificity was demonstrated with the latter organisms. Serums from the animal that received *Esch. coli* failed to be active against cells treated with the gram positive bacterial products and only feebly or not at all with those treated by homologous or heterologous *Esch. coli* extracts.

Adsorption experiments.—Adsorption studies of sera containing the nonspecies specific antibody were done using several organisms and also erythrocytes exposed to their products. The results using human serum MS 181 are summarized in table 7. Experiments done with other human serums and other strains of the same species of organism were similar. Three ml of the serum diluted 1:10 were treated with the organisms derived from 500 ml of tryptose broth culture in the cold for 18 hours. A similar volume of serum was also exposed for 2 hours in the cold to two 1.0 ml portions of sheep erythrocytes treated exactly as were those used in the test.

The data reveal that the NSS antibody was removed easily by adsorption to group A streptococci, to micrococci, or to erythrocytes treated with group A streptococcal filtrate. The latter preparation was not run in tests with micrococcus or *Esch. coli* treated cells. The anti-NSS was not adsorbed by *Esch coli*.

In other similar experiments not presented in detail the antibody was also removed by suspension of a bacillus sp. and of a nonhemolytic streptococcus and

TABLE 6.—*Hemolysis of sheep erythrocytes treated with various bacterial filtrates and acid extracts in presence of serum from immunized rabbits.*

Erythrocytes exposed to 1.2 dilution of	Hemolysis of treated sheep erythrocytes in the presence of serum from rabbits immunized with:																					
	Group A streptococcus—"Banks" Serum dilution							*M. pyogenes* var. *aureus*, no. XX152 Serum dilution							*Esch. coli*, 40069 Serum dilution							
	4	8	16	32	64	128	256	4	8	16	32	64	128	256	4	8	16	32	64	128	256
Group A streptococcus filtrate "Banks"	+++	+++	+++	+++	+	0	0	+++	+++	+++	++	+	0	0	0	0	0	0	0	0	0
M. pyogenes var. *aureus*, XX152, extract	+++	+++	+++	+++	+++	+++	0	+++	+++	+++	+++	+++	++	0	0	0	0	0	0	0	0
M. pyogenes var. *aureus*, 42800, extract	++	++	++	++	++	+	0	++	++	++	++	++	++	++	0	0	0	0	0	0	0
Esch. coli, 40069, extract	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Esch. coli, 37965, extract	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0	0	0	0	0	0

+++, maximum hemolysis.

TABLE 7.—*Results of adsorption of serum by several organisms and by erythrocytes treated with streptococcal filtrate on the content of anti-NSS.*

Serum MS 181 adsorbed with:	Sheep erythrocytes treated with:																					
	Group A streptococcal filtrate Serum dilution						*M. pyogenes* var. *aureus*, extract Serum dilution						*Esch. coli*, extract Serum dilution									
	10	20	40	80	160	320	10	20	40	80	160	320	10	20	40	80	160	320				
Normal sheep cells	+	+	+	+	0	0	+	+	+	+	+	0	0	0	0	0	0	0				
Group A streptococcus	+	+	+	+	0	0	+	+	+	+	0	0	0	0	0	0	0	0				
M. pyogenes var. *aureus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
Esch. coli	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+				
Erythrocytes treated with filtrate of group A streptococcus	+	+	+	+	0	0	+	+	+	+	+	+	—	—	—	—	—	—				

+++, maximum hemolysis.

partially by one of enterococcus.

Another similar experiment demonstrated that the anti-NSS in serum obtained from rabbits immunized by injection of group A streptococcus and micrococcus were also removed by adsorption with erythrocytes treated with a streptococcal filtrate.

DISCUSSION

Hemagglutination or hemolysis of erythrocytes exposed to bacterial substances have proved to be useful immunologic tools for the study of infection by *H. influenzae*,[7] *H. pertussis*[8] *Myco. tubercularis*,[1] *Past. tularensis*,[9,10] *Candida albicans*,[11] actinomycetes,[12] *Esch coli*,[5,6] *S. typhii*[13,14] *Pfeifferella*,[15]

7. Keogh, E. V., North, E. A. and Warburton, M. F. 1947, Haemagglutinins of the Haemophilus group. Nature, **160**: 62.
8. Keogh, E. V. and North, E. A. 1948, The haemagglutinin of *Haemophilus pertussis*. I. Haemagglutinin as a protective antigen in experimental murine pertussis. Australian J. Exper. Biol. & Med. **26**: 315–322.
9. Alexander, M. M., Wright, G. C. and Baldwin, A. C. 1950, Observations on the agglutination of polysaccharide-treated erythrocytes by tularemia antiserum. J. Exper. Med. **91**: 561–566.
10. Wright, G. G. and Feinberg, R. J. 1952, Hemagglutinins by tularemia antisera: Further observations on agglutination of polysaccharide-treated erythrocytes and its inhibition by polysaccharide. J. Immunol. **68**: 65–71.
11. Vogel, R. A. and Collins, M. E. 1955, Hemagglutination test for detection of *Candida albicans* antibodies in rabbit antiserum. Proc. Soc. Exper. Biol. & Med. **89**: 138–140.
12. Yokoyama, Y. and Hata, T. 1953, Serological studies on actinomycetes. II. On the chemical properties of the somatic antigens and on the hemagglutination reaction. J. Antibiotics, **6**: 80–86.
13. Spaun, J. 1951, On the determination of Vi-antibodies by haemagglutination. Acta path. et microbiol. **29**: 416–418.
14. Staack, H. H. and Spaun, J. 1953, Serological diagnosis of chronic typhoid carriers by Vi haemagglutination. Acta path. et microbiol. **32**: 420–423

diphtheria antitoxin,[16] and others.[17,18]

The present investigation was planned for the purpose of applying similar methods to the study of group A hemolytic streptococcal antigens and antibodies. Hemolytic and hemagglutination reactions were readily obtained with streptococcal filtrates but it was soon learned that these reactions were not specific for group A streptococci. Immunologically similar erythrocyte sensitizing substances were readily obtained from many gram positive but never from any gram negative bacteria.

Two previous investigators have described results obtained with streptococcal materials which suggest that they were dealing with the same antigen.[19,20] Their procedure failed to demonstrate definite antistreptococcal antibodies, since an antibody response was not demonstrated in infected human beings, but they failed to recognize the nonspecies specific aspects of the reaction. Another study of hemagglutination of tannic accid treated erythrocytes exposed to M-rich extracts of group A streptococci was hindered by the frequent presence of non-type specific agglutination which may well have resulted from the presence in the extracts of this same nonspecies specific substance.[3]

This report describes in some detail many of the properties of the active material (NSS). It appears in considerable amounts in culture filtrates of many gram positive bacteria and may be readily extracted from these same organisms by exposure to isotonic acid solutions at the temperature of boiling water.

M. pyogenes var. *aureus*, hemolytic, streptococci of groups A, C, and G, *Str. viridans*, enterococcus, bacillus sp., and pneumococcus were all found to be potent sources of NSS. An extract of one strain of clostridium sp. which did not contain NSS was the only failure among the gram positive organisms tested.

Several strains of *Esch. coli*, proteus sp., and pseudomonas sp., and one salmonella sp. and one gonococcus were studied and none formed the active material.

Heterophile antigens shared by more than one species of microorganisms are well known. Most widely distributed is the Forssman antigen.[21,22] The substance described here is not related to this factor since the antibody in these animal and human serums containing anti-NSS was not removed by adsorption with sheep erythrocytes.

Other heterophile bacterial antigens have been described. Best known, because they have clinical significance, are those common to certain strains of proteus and rickettsia, and to brucella

15. Boyden, S. V. 1950, Adsorption by erythrocytes of antigens of *Pfeifferella mallei* and *Pfeifferella whitmori*. Proc. Soc. Experi Biol. & Med. **73**: 289–291.
16. Fisher, S. 1952, The estimation *in vitro* of small amounts of diphtheria antitoxin by means of a haemagglutination technique. J. Hyg. **50**: 445–456.
17. Hayes, L. 1951, Specific serum agglutination of sheep erythrocytes sensitized with bacterial polysaccharides. Australian J. Biol. & Exper. Med. **29**: 51–62.
18. O'Connor, J. L. and MacDonald, J. M. 1950, Excretion of specific antigen in the urine in Tsutsugamushi disease (Scrub Typhus). Brit. J. Exper. Path. **31**: 51–64.
19. Kirby, W. M. M. 1951, Hemagglutination reaction in streptococcal infections and acute rheumatic fever. Proc. Soc. Exper. Biol. & Med. **78**: 519–522.
20. Harris, T. N. and Harris, S. 1953, Agglutination by human sera of erythrocytes incubated with streptococcal culture filtrates. J. Bact. **66**: 159–165.
21. Shorb, M. S. and Bailey, G. H. 1934, Heterophile antigen in various bacterial species. Am. J. Hyg. **19**: 148–183.
22. Buchbinder, L. 1935, Heterophile phenomena in immunology. Arch. Path. **19**: 841–880.

and *Vibrio cholerae*.[23] Others include Type 2 pneumococcus and Type B Friedlander's bacillus,[24] type 2 pneumococcus and *Pasturella cuniculicidam*[25] enterococcus and shigella,[26] type 29 and 6 pneumococcus and type B influenza bacillus,[27] and type 3 pneumococcus and gonococcus and meningococcus.[28]

Insofar as is known to the author, none of these was sharply limited to gram positive organisms as is the NSS. No precedent for an antigen associated with the staining characteristics of microorganisms of many species appears to have been established. An exception may be the nucleoprotein antigens common to streptococci, pneumococci, and, to a less erextent, to micrococci.[29] These would seem to be different from the NSS since they were soluble in dilute alkali and precipitated in the presence of acid. These properties are quite unlike those of NSS.

The chemical nature of the NSS is presently not completely defined. It is heat stable in acid solutions, appears in extracts of bacteria prepared at low pH, is not dialyzable, and is resistant to tryptic digestion. Alcoholic precipitates of phenol extracts of organisms contain large amounts of it.

These observations suggest that the NSS is a polysaccharide as are nearly all of the heterophile antigens listed above. The nucleoproteins studied by Lancefield are an exception. Earlier studies of hemagglutination and hemolysis of erythrocytes treated by bacterial products (4 through 17) have indicated that the active substance is usually a polysaccharide.

Recently, Pakula,[30] using the technique of hemagglutination and bacteria disrupted by the proteolytic enzyme of *Streptomyces albus*, has described a heterophile polysaccharide antigen present in micrococci and group A streptococci. Independent observations in this laboratory have confirmed the existence of this substance, but is it believed to be derived from the streptomyces filtrate rather than from the treated organisms. These studies will be reported elsewhere.

SUMMARY

Culture filtrates and extracts of certain bacteria contain a nonspecies specific substance which is capable of preparing erythrocytes for hemagglutination or hemolysis in the presence of complement and certain human and immune animal serums.

The active substance was obtained from gram-positive, but not gram-negative organisms. It was nondialyzable, heat stable in acid solutions but labile at neutrality and was not destroyed by tryptic digestion.

The substance present in human and animal serums which reacts in this system has the attributes of a true antibody.

23. Elberg, S. 1952, The Brucella in Bacterial and Mycotic Infections of Man, Philadelphia, J. B. Lippincott Co.
24. Landsteiner, K. 1945, The Specificity of Serological Reactions, Cambridge, Harvard University Press.
25. Dingle, J. H. 1934, The serological specificity of bacterial carbohydrates with special reference to type II pneumococcus and a heterophile strain of *Bacterium lepisepticum*. Am. J. Hyg. **20**: 148–168.
26. Ingalls, M. S. 1937, A contribution to the nature and origin of natural agglutinins. J. Immunol. **33**: 123–138.
27. Zepp, H. D. and Hodes, H. L. 1943, Antigenic relation of type B *H. influensae* to type 29 and type 6 pneumoccoci. Proc. Soc. Exper. Biol. & Med. **52**: 315–317.
28. Miller, C. P. and Boor, A. K. 1934, The carbohydrates of gonococcus and meningococcus. I. The alcohol precipitable fraction. J. Exper. Med. **59**: 75.
29. Lancefield, R. C. 1925, The immunological relationships of *Streptococcus viridans* and certain of its chemical fractions. II. Serological reactions obtained with antinucleoprotein sera. J. Exper. Med. **42**: 397–412.
30. Pakula, A. 1952, Rosnorodnosc weilocukrow u paciorkowcow hemolitycznych analiza metoda hemaglutynacji i precypitacji. Medycyna Doswiadezalna i Mikrobiologia, Warsaw, **4**: 197–216.

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